

EDUCATION, VALIDATION OF THESIS
STIPULATION PROCESS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1967

ACKNOWLEDGMENTS

The author is indebted to Dr. Arthur G. Telenius, his major professor, for encouragement, advice and patience throughout the course of this work and especially for his assistance in preparing this document.

The author is also highly grateful to Dr. Elton R. Stange, Dr. Francis F. Neely, Dr. James A. Lindsay, Dr. Richard E. Gray, and Dr. Chung K. Hsieh for their time, patience and extremely valuable advice.

The author is very grateful to Larry Miller for his advice and assistance on computer-related problems, to R. E. Orin for the fabrication of different views, and to John and Gerda Wilson for their efficient technical support.

Finally, the author deeply acknowledges the constant support and encouragement from his wife Kayden, and the patience and support of his parents, brother and sister.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

ANALYSIS, VALIDATION OF THEORY,
AND APPLICATION OF THE
STERILIZATION PROCESS

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August 1967

Chairman: Irving A. Trilling
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Heat inactivation of bacterial spores is of critical importance in assuring the stability and safety of sterilized products. Biological indicator units (BIUs) offer considerable potential for microbiological validation of thermal processes by providing direct evidence of the lethality achieved. Large discrepancies between predicted and observed numbers of surviving spores are often observed when traditional kinetic models are used to predict the number of survivors.

The traditional kinetic models do not account for some factors that have been found to affect the number of surviving spores. These are activation, injury, and the possible existence of a less heat-resistant fraction.

An improved mathematical model for the prediction of the effects of heat treatments at isothermal temperatures on bacterial

spores that failed both thermal collection, inactivation, and the presence of a less heat-resistant fraction was developed and tested. The model was validated by applying systems analysis to population dynamics of bacterial spores. A three-axis fluid chamber was used to perform both novel laboratory experiments with *Bacillus subtilis* spore suspensions. Results of these experiments were used to determine various variables in the mathematical model. The model was then tested under nonisothermal conditions, and showed close agreement between practical and calculated number of survivors from a lethal processed temperature profile.

Improved biological indicator units were designed and fabricated, and their performance compared to that of traditional units used in commercial practice. The fabricated units offered improved heat transfer and mechanical properties that allowed the spore suspensions to experience the actual thermal history of the product with added protection from pressure changes.

These results introduce new improved methods for the design, calculation, and validation of thermal sterilization processes. Specifically, they establish a procedure by which the sterilization effectiveness of a thermal process can be tested objectively.

INTRODUCTION

Heat inactivation of bacterial spores is of critical importance in assuring the stability and safety of canned foods and pharmaceuticals. Exposure to the high temperatures of commercial sterilization processes is detrimental to the nutritional and organoleptic characteristics of foods and to the stability of drugs. Consequently, a heat treatment must be severe enough to accomplish sufficient inactivation of bacterial spores, while avoiding excessive exposure of the product to high temperatures. Improved methods for design and verification of thermal processes are needed to ensure the precision and safety of these heat treatments. Development of improved methods requires an understanding of the engineering sciences of heat transfer and the available kinetics of thermal inactivation of bacterial spores.

The design of thermal sterilization processes has been traditionally based on the assumption that thermal inactivation of bacterial spores can be modeled as a single first-order reaction. In other words, it may be described by a straight line when the logarithm of the number of surviving spores is plotted against time of exposure to a constant lethal temperature (quarter curves). However, actual survivor curves plotted from

laboratory data frequently reveal considerable deviation from a simple straight line during early periods of exposure. This is a consequence of additional, concurrent reactions such as heat activation, injury, and preliminary inactivation (early inactivation of less heat-resistant fractions).

Deviations from a straight line model often make it difficult to test or validate a thermal process microbiologically. Plate counts from a given organism subjected to a variable temperature history in a commercial process often do not agree with predicted results from the simple, first-order reaction model. Also, the increasing use of high temperature-short time sterilization processes with aseptic packaging systems requires better kinetic models because the accelerated reactions may render uncertain the degree of inactivation achieved during relatively brief exposures to lethal temperatures. Therefore, an improved kinetic model should include all simultaneous, temperature reactions in order to predict accurately the shape of survival curves obtained from laboratory data.

Biodefined units (BDUs) offer considerable potential for microbiological validation of thermal processes to support the shipment of safe, thermally-processed products by providing direct evidence of the lethality achieved. Two important requirements for adequate performance of BDUs are a) the system suspension itself within a unit must experience the full temperature history as the coldest place in the product,

and b) the material used for the IIR should be innocuous to the agent. Therefore, a bioindicator unit should offer the least possible resistance to heat transfer from the product while ensuring self-insulation from heat that would be conducted from the use wall-stove interface, and it should be compatible to the indicator characteristics contained therein.

The need for more complete kinetic details and improved bioindicator units for the biological validation of thermal sterilization processes led to the objectives for this work. The objectives were a) to develop and test an improved mathematical model for predicting the number of survivor spores in response to isothermal heat treatment, taking into account the concurrent reactions of activation and inactivation in addition to inactivation, and b) to design and fabricate bioindicator units with improved heat transfer characteristics and to compare their performance with that of traditional units used in commercial practice.

Conceptually, this research started by verifying reports on discrepancies found between the final number of survivor spores in plastic kits predicted by Bigelow's model and observed by microbiological enumeration for a given temperature history. In a second stage, the relative importance of heat transfer on the performance of IIRs was investigated experimentally. This involved the design, fabrication and testing of kits with different heat transfer characteristics. The results led to the

concludes that heat transfer cannot completely explain the discrepancies between calculated and measured numbers of surviving spores after a heat sterilization process and that the conventional model utilized to describe the kinetics of heat-treated spore inactivation (Bigelow's model) is not precise enough for the utilization of thermal sterilization processes.

To develop systematically an improved kinetic model of spore inactivation, consideration was given to the fact that in addition to inactivation, which is a transformation of viable spores resulting from viable to dead in the enumeration medium, other transformations may occur which are not considered in the models utilized in practice for design and validation of thermal sterilization processes. The main factors affecting the number of surviving spores in response to a thermal treatment have been identified as activation, injury, the existence of less heat-resistant fractions, and alternative germination mechanisms. Activation is a transformation of viable, dormant spores resulting from germination and growth in an enumeration medium. Injury is a transformation of spores that modifies their requirements for germination and growth.

The hypotheses tested in this research were a) the descriptions of inactivation, activation and injury as simultaneous and independent first order reactions, and b) the existence of less heat-resistant fractions of a spore population, and c) the

existence of an alternative germination system whose activation by heat killed *E. coli* spores is provided the incubation temperature is 32 deg. C. These hypotheses led to a conceptual model of a population of bacterial spores treated at lethal temperatures. The conceptual model was analyzed to derive a mathematical model which, in turn, was used to predict the dynamic responses of spore populations to lethal temperature exposure. In the case of constant lethal temperature, the mathematical model was solved to obtain formulas for the responses (population dynamics), which consisted of exponential functions of time involving parameters corresponding to initial conditions and rate constants.

To determine values for parameters of the model, a series of isothermal experiments was performed where *E. coli* spores were exposed to lethal temperatures. The survivors at successive times were enumerated microbiologically, and the experimental responses were fitted to the response formulas of the model using a nonlinear regression technique. Temperature dependence of rate constants was found to be well described by Arrhenius equation. To validate the model, a dynamic experiment was performed where a spore suspension was subjected to a variable temperature history that also was used to generate corresponding responses of the model by simulation. Model generated responses were compared with the results of microbiological enumeration of survivor spores in samples taken during the experiment.

The results show the capability of the new model to describe well the dynamics of a population of bacterial species exposed to both constant and transient lethal temperatures.

LITERATURE REVIEW

Study of the biological validation of thermal sterilization processes using microorganisms has important physical and biological components. Heat transfer plays an important role in the ability of a microorganism unit to follow the temperature history of a product, and the thermobacteriological behavior of a given temperature used as the indicator determines the number of survivors to lethal heat treatment. This review starts with information pertinent to system analysis and experimental design corresponding to important aspects of biological validation of thermal sterilization processes.

In the first section of this chapter, use of microorganisms in the validation of thermal process is discussed, with emphasis on basic principles, performance, and lessons of practical experience. The state of the art in validation of sterilization process is such that in spite of the potential importance of RBE, disagreement between the number of survivors determined from the transitional model and microbiological enumeration, often by several orders of magnitude, is an important restriction on their use. The next extensive section presents biological aspects of spore population dynamics under lethal treatment temperatures. The complexity of biological materials requires careful consideration of information available on the species,

transformations and interactions involved. This information is the foundation for systematic development of conceptual and mathematical models that allow analysis for better qualitative and quantitative understanding of the behavior of sparse populations. The last section of this review is a brief examination of the most important concepts of system analysis applied to biological systems.

Use of Microbiological Methods in the Evaluation of Sterilization Processes

The use of microbiological methods in testing the effectiveness of commercial sterilization of food and pharmaceutical products has been difficult and, at best, only marginally effective. Historical efforts made use of incubated peak plates, in which product samples were inoculated with large concentrations of heat resistant spores and incubated after processing in order to relate the fraction of product aseptical to the number of surviving spores. More recent methods use small vials containing fresh spore suspensions that are inoculated into product vials during a sterilization process. The vials are subsequently recovered, and the survivors are enumerated microbiologically to estimate the extent of product sterilization. However, in practice, the number of survivors counted after exposure to the sterilization process seldom agrees with

the number predicted by Papirer's kinetic model of the indicator spore population subjected to the temperature history of the process.

Process validation has been a topic of particular interest in the pharmaceutical industry over the last ten to fifteen years. "Process validation is establishing documented evidence that a process does what it purports to do" (Agallucci 1984). The same author reported participating in validation studies of steam sterilization which reduced cycle time by two-thirds while still maintaining an overkill cycle, thus suggesting that very large safety margins exist in commercial sterilization processes, with consequent waste of time and energy and negative effects on product quality.

Biological indicators are important tools in the validation of sterilization processes. These are standardized preparations of specific microorganisms of known resistance to a sterilization process agent. In the parent form of biobactericides, microorganisms are added directly to the product being sterilized. When this approach is not practical, an alternative is to add the organisms to carrier materials, or containers, known as biobactericide vials (BIBs). In appropriate applications of this type of indicator, BIBs should have a time constant of heating and cooling that allows the contents to follow closely the thermal history of the product (Jenney, 1983).

There are two categories of tests by which the efficacy of sterilization can be assessed: either the product, or a statistically significant sample of it, is directly tested for the presence of viable microorganisms, or the time-temperature conditions of the sterilization process are monitored and analyzed in terms of existing knowledge about the behavior of microorganisms under the stress conditions of sterilization. In this report, BIA have the advantage over other methods of responding to all the component factors of a process. The essential requirement for effective performance of a BIA is that its behavior under stress must be predictable [Ramsell, 1980]. Pflug and Doffing (1986) published a review of the use of BIA in the pharmaceutical and medical industry. A biological indicator system is defined to consist of microorganisms, usually bacterial spores, procedures and equipment for preparing the BIA, and the measurement procedure for determining the level of sterilization achieved at a specific location in the product.

BIA that use living bacterial spores as sensing units cannot be considered a calibration standard. From a standpoint of sterility, BIA must be calibrated to a laboratory entry system that are based on physical standards. Pflug and Doffing (1986, pages 100-101) also mention that there is general understanding and agreement among scientists working on sterilization in the pharmaceutical industry and in regulatory agencies that "the design of a sterilization process for a

product that will be marketed as sterile must be based on the number of microorganisms on or in the product (Stoburton) ¹⁰ The probability of a nonsterile unit (NTU) on the order of 10^{-6} is widely accepted as a standard target for most tamely-sterilized products. The conclusion is that BPs, with their inherent variability, are reasonably accurate and reproducible in measuring a sterilization effect when properly used.

Ryan and Drai (1980) published a review on the best principles and applications of bioreactors. Bioreactor systems should be prepared to offer a realistic challenge to the process. The approach is to set the concentration of the indicator organism to the number of the material to be sterilized. The top stage of the indicator concentration to provide a safety factor. Care should be taken not to create situations that would add resistance to penetration of heat from the sterilizing medium. Brack (1984) published an analysis of the probabilities of survivors which showed the advantage of BPs over traditional sampling methods. The review was underlain by a series of hypotheses originated from concentrated, large volume, parenteral solutions. The main conclusion of Brack's study is that the simplest way to achieve the desired probability of 10^{-6} (practically impossible by throwing sample size) is through the use of BPs.

Flexible BPs are described and discussed by several authors (Jones et al., 1980; Pflug et al., 1980 and Pflug, 1982). Jones

et al. (1988) describe the use of film to measure the effect of pill weight on the release delivered to grain blots using a statistical report. Pflug et al. (1988) compared differences in the values of leachability calculated by the general method (Jalil and Green, 2011) and described by microbiological experiments of various species of *E. faecalis*. They found that to be far larger than variations due to experimental error (± 1.2 vs. ± 0.2 to ± 0.8). Average differences between calculated and measured leachability were significant as long as 4-4 min. The authors said the conclusions that could be made were limited due to the complexity of the overall measurement problem.

The United States Pharmacopoeia (USP) has supported and encouraged studies aimed at setting standards for film. Cooper (1992) described a series of reports and assessments from the USP that have been published. Several of the reports reveal the need to improve the quality of films commercially available (Rogers, 1992; Beck, 2002). Smith et al. (1992) published a report on three different commercially available biological indicator (BIO) strips. They are type and the manufacturer's specifications. Gillis (1992) tested six commercially available biological systems. He found that not all of them were properly labelled and that the claims of some manufacturers were not sustained. Rogers' (1992) paper on technological evaluation of thermal process design presents the approach of the food-processing industry, which is based on the inoculated

pick system and on the usual redox system. The product-fractional response method for evaluation of sterilization processes was offered by Gavett et al., (1971). In this method, neutralized fibrous product is exposed in a production vessel to various fractions of a proposed or existing sterilization cycle time, and the surviving bioburden is measured for each fraction of the cycle to determine the time necessary to achieve sterilization. The use of *B. pterodispermophilus* as a biological indicator was investigated by Fleming et al., (1972). The effect of storage conditions on this using *B. pterodispermophilus* was explored by Birch et al., (1973) over a 1.5 year period that demonstrated the decline in resistance over time of several commercially available BIs.

Bacterial Spore Inactivation

All species of bacterial sporeformers are in the family Bacillaceae, divided into the five genera *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Desulfotomaculum*, and *Sporosarcina*. Since the tremendous discovery of bacterial spores by Koch and others in 1876, a considerable amount of research on spores has been done. Studies on thermal inactivation of bacterial spores are intimately related to studies on disinfection. In 1937, Brody and Paul published an extensive paper on disinfection that established some of the basic criteria and procedures for

experimental study of disinfection: the effects of time, concentration and type of disinfectant, temperature, and type of test bacteria on the number of survivors were carefully examined, and several rules for testing the efficacy of disinfectants were proposed. The authors were aware of the work of Arrhenius, and they recommended the use of physical-chemistry in the study of disinfection.

In 1916 Orsk published a paper on disinfection, where hot water was used as a disinfectant. The author reviewed the results published by Emory and Paul (1907) and showed that disinfection proceeded in an orderly manner and that the rate decreased as the number of survivors became less. Rules were made that, in previous work with sodium chloride and glucose, the concentration of spores remaining above various logarithmically with time and that the number of spores destroyed per unit time was proportional to the concentration of viable spores at that moment. Analysis of the data published by Emory and Paul (1907) showed the same relationship. Experiments were conducted to determine first-order rate constants, and an attempt was made to use Arrhenius' equation between k and $10 \log 10 C$. Some deviations from logarithmic linearity were found. Millions of the disinfection of proteus when heat treatment was noted, together with very high values of the activation energy. The conclusion was that disinfection, whether by disinfectants or by

heat, could be considered analogous to a chemical reaction, the rate of which is controlled by conditions of temperature and concentrations of bacteria and disinfectant.

Wiggins (1924) studied the protective effect of sodium chlorate on bacterial spores heated in gas liquid. This work related directly to the canning industry, dealing with the effect of product composition on spore inactivation. The protective influence was apparent at sodium chlorate concentrations of 4 to 3.0%. Higher concentrations had no protective effect and even produced a killing effect. The logarithmic character of spore inactivation also was noted, and the results were presented in semi-logarithmic form. The importance of environmental composition was also studied by Smith and Whittier (1925), and survival curves were found to be logarithmic in nature. The effect of alkalinity on rate constants was such that doubling the concentration of alkali increased the value of rate constants about tenfold, and increasing the temperature from 50 to 55 deg. C increased the value of rate constants nearly 100-fold. The effects on rate constants of age and initial concentration of the spores were studied, and aging was found to decrease while concentration increased the values of rate constants.

The importance of preventing inactivation of bacterial spores for the canning industry led to the development of empirical methods. The thermal death point was defined as the time

interval at a given temperature necessary to destroy a specified initial concentration of spores in a mixture of known pH, and a method was proposed for determining the thermal death point (Bigelow and Eddy, 1931). Later, the logarithmic nature of thermal death-time curves was finally taken into consideration, and the use of semi-logarithmic graphs was proposed for determining the time necessary to destroy a certain fraction of the initial viable spores (Bigelow, 1921).

The general method (Bigelow et al., 1939) for design and evaluation of thermal processing foods is a graphical integration procedure for determining the lethal effects of different time-temperature regimes at the coldest spot in a container. Decepton said this method easy to use today, but it was tedious and time-consuming when first introduced. Ball (quoted in Martin, 1944) developed a mathematical method for calculations pertaining to thermal processes that is frequently used today (Formis's Method). Ball was a strong proponent of using mathematical tools in practice, his Formis method is based on a logarithmic approximation to the heat penetration curve in a finite cylinder (cylindrical can). For the same purpose, Martin (1944) developed a mathematical method that considers all points in the container. This was an improvement over Ball's Formis method, which considered only the can center as the slowest-heating point. Therefore, by 1939, several methods were available that allowed calculations for thermal sterilization

processes. This subject was thoroughly analyzed and discussed in two books that may be considered classics; they provide ample information on the theoretical and practical aspects of thermal process design (Ball and Olson, 1987, and Steele, 1988). Ball (1988) also discusses the relationship between thermal processes and the organoleptic characteristics of canned products, and he recommended high-temperature short-time treatments of natural and forced convection-heating foods. The above mentioned works form the basis for system design and evaluation of thermal sterilization processes, and they rely on general first-order kinetics to describe inactivation of bacterial spores.

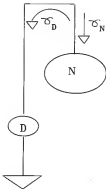
Classic models

The traditional view of bacterial spore inactivation at a constant initial temperature may be represented by the model in Figure 1 composed of a store, N_1 , of viable spores, an inactivation transformation, I , and a sink (triangle) for dead or heat-killed spores. In Figure 1, the variables studied by identity flow related to these processes. An analysis of the model is now presented.

The description of the store is

$$\frac{dN_1}{dt} = -I \quad (1)$$

Figure 11 Conceptual model corresponding to the single transformation approach to sperm functionalization



linearization as an algebraic transformation [5]

$$\dot{V}_B = k_B (1 - V_B) \quad (1)$$

where k_B is the rate constant and t is time. The model has a single node, at which the sum of incident flows equals zero

$$-V_B + \dot{V}_B = 0, \quad \text{or} \quad \dot{V}_B = V_B \quad (2)$$

Substitution of equations 1 and 2 yields the overall model of bistochastic as analogous to a first order reaction

$$\frac{dV}{dt} = -k_B V \quad (3)$$

Since the overall number of viable species is the solution of (3) is as follows

$$V = V_0 \exp(-k_B t) \quad (4)$$

The logarithmic form of the solution often is used,

$$\ln(V/V_0) = -k_B t, \quad \text{or}$$

$$\ln(V/V_0) = -t/\lambda \quad (5)$$

where λ , called reduction time, is the time interval required to reduce the population of viable species to one-tenth of its former value (note that $\lambda = \ln(10)/k_B$). Figure 1 is a

Figure 2. Semi-logarithmic server curves. Backoff reduction time, R , is the time required by the server curve to change by one order of magnitude.

ing summer



Lunar

on a logarithmic plot of the inactivation of a bacterial spore population, the thermal death time (TDT) curve, where θ is indicated to be the interval required for the TDT curve to advance one log cycle...

In practice, thermal processes are often described by their lethality. For a constant treatment temperature, lethality F is defined to be the time required for a specified reduction of the population of viable spores. From Equation 4, lethality at a constant temperature is expressed by:

$$F = \theta \log(n_0/N) \quad \text{..... (1)}$$

Since θ is temperature dependent, F is expressed, by convention, for a reference temperature of 121.1 deg. C (250 deg. F):

$$F_{121} = \theta_{121} \log(n_0/N) \quad \text{..... (2)}$$

For instance, if the value of the decimal reduction time at 121.1 deg. C is 3 min and the desired reduction is the number of viable spores is 9 orders of magnitude, the lethality of the 10 D min.

The empirical expression used in practice to describe the variation of θ with temperature is

$$\log(\theta_{T_2}/\theta_{T_1}) = (T_2 - T_1)/z \quad \text{..... (3)}$$

where T_1 and T_2 are two different isothermal temperatures and ΔT is the temperature increment required to reduce the value of the k by one order of magnitude, as shown in Figure 3.

For a variable temperature process, $\log(k_2/k_1)$ is determined by dividing the thermal history into small time intervals wherein the temperature is considered constant. The effect of temperature during an interval ($T_2 = T_1 + \Delta T$) on the number of surviving spores follows from equation (8)

$$\log(k_2/k_1) = (k_2 - k_1)/k_1 \quad (9)$$

If the time increment is of differential magnitude, the total lethal effect is obtained by adding the contributions of the differential treatment:

$$\log(k_2/k_1) = \int_0^t \frac{dt}{k(T)} \quad (10)$$

From the description of temperature dependence of k (equation 8) and using the 121 $\pm$ deg C (250 $\pm$ deg F) reference temperature, the value of $k_0(T)$ is

$$k(T) = (k_{121} - 1) \cdot 10^{\frac{(T-121)z}{D}} \quad (11)$$

Figure 1. Thermal destruction curve.

log 0



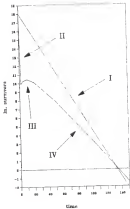
temperature.

The terms defined $-D$, I and I_{∞} are commonly used to define the heat resistance of bacterial spores and the lethal effect of sterilization processes on them.

Figure 4 presents an example of the complex behavior that may be observed during thermal inactivation of bacterial spores; it includes an initial decline in the number of survivors due to preliminary inactivation, a subsequent rise in the number of survivors, and, finally, the prolonged inactivation phase. The straight line (1) corresponds to the simple, traditional model (Equation 4) where inactivation is assumed to be the only significant reaction. It also corresponds to the limited practice of defining the kinetics of spore sterilization at constant lethal temperatures by fitting Equation 4 to only two points, at time equal to zero and at the time when the population has been reduced to one viable individual per unit volume. In this case, the most probable number (MPN) of survivors technique is used to enumerate surviving spores. Use of the simple straight line model (Equation 4) may lead to large errors in the estimation of bacterial spores that will survive a lethal heat treatment or the heat treatment necessary to render a sterile end use product.

The modeling of complex systems of irreversible first-order chemical reactions was investigated by Lee (1970). A lumped structure allowed for the synthesis of kinetically constant structures of reaction. That paper dealt with organic chemical

Figure 4. Deviations from logarithmic linearity during inactivation of bacterial spores. I corresponds to the logarithmic behavior, II to preliminary inactivation of less heat-resistant fractions, III to the region controlled by activation and IV corresponds to predominant inactivation.



mechanisms. Prelog and Ingley (1970) presented a review paper on models of disinhibition kinetics. The proposed models share a common reaction mechanism. Shull et al., (1980) also proposed a common reaction mechanism for sperm activation, postulating the existence of activated, inactivated, and inactivated states. As a consequence, activation and inactivation would be dependent, i.e., the sperm must be activated first and then inactivated. No biochemical basis was provided for this assumption.

More recently, Gellera and Burns (1984) developed a kinetic model for *S. thermophilus*, that also assumed sequential reactions. Avasthi and Lund (1985) published a paper on procedures for properly calculating kinetic parameters from experimental data. Both *de Wit* (1985) investigated the thermal characteristics of sperm clumps. The increase in life-span of a clump was proportional to the logarithm of the number of viable sperm within the clump. *de Wit* and *de Wit* (1986) analyzed thermal death rates of bacterial spores. They found that a normal distribution of individual life-spans produced a thermal death rate of a population of spores in the same form as Equation 4. The life-span of a spore was defined as the length of time the spore remained viable in a given temperature. Each spore was assumed to have a specific life-span, and a spore population was assumed to have some distribution of life-spans. The authors recommended supplementing the first-order reaction

rate well with probability theory. Berry and Drobner (1982) compared the latencies of thermal sterilization processes obtained from thermal histories using Bigelow's model and from microbiological enumeration of survivors. They found differences as large as 2.2 min. (the calculated value was larger than the microbiological measurement), and they conclude that the two sterilization values are not directly comparable.

Options for the experimental study of inactivation kinetics

The need for precise control of exposure to lethal temperatures in the measurement of thermal inactivation kinetics has led to considerable research with alternative laboratory equipment and methodology. Delisari and Latcha (1982) used a three-neck flask reactor to measure survival curves for a treatment temperature of 100 deg C. The survival curve for *B. pasteurizationis* showed a maximum after 4 hr. of exposure (see Fig. 1-19b, footnote), when ca. 10% of the spores found by microscopic count were recovered. Burton et al., (1971) studied the thermal death kinetics of *B. pasteurizationis* at a range of temperatures (197). The capillary tube method was compared with a direct heating BT plate. Davis et al., (1977) analyzed the heat transfer of the capillary tube method to determine possible correction factors for BT treatments. Davis et al., (1977) found that capillary tubes could be used up to 140.6 deg. C

before dispersing from incubation' kinetics. A continuous flow apparatus for the determination of heat-labile toxin kinetics was published by Dougan et al., (1975) and Srinivas et al., (1980). Dougan (1975) proposed the use of a warming tube technique where an aliquot of spore suspension was injected into a heating coil at the treatment temperature. The volume of the heating coil was 10 times larger than that of the spore suspension, this permitted very fast temperature rise (the change in the reactor internal temperature is of the order of 10 and process temperature is regulated to a few seconds). Muller and Scharfetter (1981) developed a solid heating block system for determining heat resistance of spores. Griffiths and Rogers (1981) proposed a self-pouch system. Smith and Shennar (1982) designed a computer-controlled reactor using an air-operated piston to move into exposure a known volume of sample.

The use of differential scanning calorimetry in studies of bacterial thermal death has been investigated by several authors (Griffin and Daniels, 1982, and Miles et al., 1984). The Densograms of highly heat-resistant spores are different from those of less heat-resistant spores. Differential scanning calorimetry may become an important research tool, since the measured changes in heat capacity are related to changes in the degrees of freedom of molecules that may be involved in

roomingness of vital spore structures. Miles et al., (1984) designed equations for differential scanning calorimetry to predict survivorship when the heating rate is a constant.

Physiology of Bacterial Spores

Spore design and evolution of thermal sterilization processes is based on the simple pseudo first-order kinetics of the inactivation of bacterial spores. In order to achieve a better understanding of the various reactions that occur during the exposure of bacterial spores to lethal temperatures, relevant aspects of bacterial spore physiology will be reviewed in more detail in the following subsections.

Spewell (1984) presented a comprehensive review of the structure and chemical composition of bacterial spores that is frequently mentioned in the literature. Reference was made to the fact that bacterial spores are complex in structure and composition, differing from vegetative cells by the presence of various additional layers, unique substances such as dipicolinate acid (DPA), and quantitative differences such as a 1000 times higher calcium concentration. The preparation of spores for analysis was discussed in detail. Bacterial spores contain about 10% nitrogen, 33 carbohydrate, 33 calcium dipicolinate, and 20 ash. Spores of all species have the same basic structure: a central core or protoplast surrounded by the plasma membrane,

gem cell wall, cortex, coats and envelopes. The gem, plasma membrane and gem cell wall constitute a condensed unit, contained and protected by the outer structures (Gierke, 1954a). The envelope may be tightly or loosely fitting. In cases where information was available, the envelope was found to be mostly protein with significant amounts of carbohydrates.

Germ coats show an interesting variety in both appearance and complexity. Three main types of layers usually can be distinguished. The most distinctive is the olefin layer, which shows a characteristic lamellar pattern. Some species have heavily ridged and warty coats, while others have simple, thin coats. Coats consist largely of structural proteins. Lesser amounts of carbohydrates and lipids are generally found, and in some species large amounts of phosphorus. Coats comprise 30 to 60% of the dry weight and up to 80% of the proteins of the germ. The major extractable protein appears to be similar in distinct species.

Destruction of germ coats is a major activity of a mother cell during spore formation. The main stages may be described briefly: first, phosphorylation of polyphosphates and, second, the degradation, modification, addition and rearrangement involved in assembly structures. Spore coats first appear when the forespore has contracted and concomitantly with cortex formation and the beginning of refractility. Completion of the coats is a late event, occurring at the same time as full refractility and

heat resistance. The cortex in a mature spore is a featureless, electron-transparent zone between the core and the outer cell wall. Spore structure is related to type I polysaccharide chains in vegetative cell walls. Some modifications unique to spores are present. 45-60% of the carboxylic acid residues link with a peptide and an amino acid part; instead they form an internal anion. The core or protoplast is a relatively "dried" cell in terms of its macromolecular constituents. Some differences exist in margins.

The properties of spore DNA are not significantly different from those of vegetative DNA. Bacillus subtilis spores contain a single complete genome, but other species may have multiple copies (Pillay-Jones and Young, 1958). The physical states of margin and low molecular weight solutes in the core depend very critically on the amount of water present, and spores have lower water content than vegetative cells.

The water activity (a_w) and density of Bacillus sporotheca-subtilis spores have been found to be 0.82 and 1.4 g/ml, respectively (density was determined along a gradient of deuterium-enriched base and car-bonyl alcohol) by Flyn and Witt (1980). Other authors have found the density to range from 1.22 to 1.34 g/ml for B. subtilis and from 1.25 to 1.35 for different kinds of B. subtilis, using gradients of water-soluble, nonaqueous and porous (Pillay et al., 1980). The nature of dehydration during sporogenesis has been studied by Witt

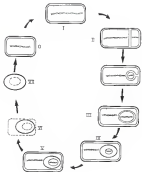
cellular. Margulis et al., (1985) used nondestructive physical techniques (measurement of the dielectric properties)

Many gram-positive bacilli (including *B. subtilis* and *Clostridium* spp.) form endospores under certain adverse nutritional conditions which no longer allow vegetative growth (Fennel, 1981). This process is called sporulation, and the cell that originates a spore is called the parent cell. Recently, sporulation of *B. subtilis* was reviewed thoroughly by Fennel (1985) and his paper is used here as the fundamental reference on sporulation. Exponentially growing (vegetative) *B. subtilis* can differentiate to form heat resistant endospores in response to starvation for carbon, nitrogen or phosphorus. Differentiation of *B. subtilis* begins approximately six hours at 40 mg. C. Population behave fairly synchronously, although there is no need for interaction within the population. The basic sequence of changes during the formation of bacterial spores is similar for all species of *Bacillus* and *Clostridium* that have been examined. The convention of numbering different sporulation stages with Roman numerals is now in general use.

The morphological changes associated with *B. subtilis* sporulation are presented in Figure 5. Sporulation can be divided into three successive phases.

a) The start of differentiation (stages 1 to 11) initiated by the formation of two chromosomes, and completed when the cell divides into two cells that differ in size. The transition from

Figure 5. Morphological changes associated with Salmonella infection.



a vegetative bacterium to a bacterium including an asymmetrically split spore septum may be considered a modified cell division. An independent factor has been demonstrated in *B. pasteurii*, and extracts of many sporoblasters possess the ability to transform vegetative cells to germinated cells having the property of sporulating endoreproductively (Delestrée, 1955).

b) In the second phase (stages II to III), differentiation becomes fixed. The two cells formed have their own genomes that may function differently. When the septum is formed at stage II, the two cells differ in size, but their contents are presumably similar. Addition of rich media at this stage causes the septum to thicken, and into cells merge as vegetative bacteria (this may not be true for all species). After stage III, a mother cell can no longer return to a vegetative bacterium upon addition of rich media, while a forespore can return to vegetative growth only by spore formation and subsequent germination. From stage III on, the forespore is no longer in contact with the mother; it is entirely surrounded by the mother cell.

c) The characteristic properties of a mature spore are developed in the third phase (stages III to VII). Penetration of the cortex and peripheral germ cell wall between the apposed membranes is defined as stage IV, and deposition of the proteinaceous spore coat around the forespore defines stage V. The processes involved in some species: Synthesis of dipicolinic

and begins at about stage IX in the mother cell. Calcium stimulates the response slightly prior to DNA stimulation. Isolated forespores have a specific facilitated diffusion system for Ca^{2+} [Leflow, 1982]. Heat resistance develops during stage V and increases gradually. Heat resistance is characterized by an increment in lethal heat treatment required to inactivate a spore, often by several orders of magnitude.

Germination is a well defined stage in the development of spore forming bacteria. It seems conversion of a resistant, dormant spore into a sensitive, metabolically active form [Davis, 1981]. It is also described as a heat labile, non-refractive, and absorbable form, yet readily distinguished from vegetative bacteria [Japan and Anderson, 1980]. The kinetics of germination of resistant spores were studied by Harg and Bolomick (1983). The overall germination process has been defined in terms of the following events: a) conversion, b) loss of heat resistance and excretion of calcium-ATP, c) loss of spores, refractivity, and its resistance [Leflow, 1981]. Leflow and Antipova (1983) developed a mathematical model of spore germination based on population dynamics. The system was defined as a first ordered sequence of four distinct phases: Activation, Triggering, Initiation, and Outgrowth. Each phase has a characteristic time lag and a characteristic rate of evolution controlled by a maturation function. The model

fitting the experimental data very closely, this work is an example of the application of system analysis of population dynamics to the study of bacterial spore germination.

Heat Resistance of Bacterial Spores

Bacterial spores exhibit the greatest thermoresistance of all living organisms. It has been stated that of vegetative bacteria, only a titrate of 10^8 or more. The range of heat resistance between spores of different species is very large (Brink and Brink, 1934). In acidophilic species heat resistance generally correlates with the average growth temperature of the vegetative form (Gerstl, 1938a). Heat resistance varies among spore crops, and it changes with composition of the sporulation medium (Brink and Brink, 1939). It is not certain which spore component or structure is the primary target of thermal inactivation (Gerstl, 1939). Ushakov et al., (1955) published a review of the subject which suggests that theories on heat resistance fall into two main classes. The first proposes that resistance results largely from partial dehydration of the protoplast. The second proposes that heat resistance depends on molecular rearrangements resulting from the presence of calcium dipicolinate, glycine, phosphoglycerate, low molecular weight basic proteins and possibly other substances that might stabilize spore components. Mechanisms discussed in more detail were as follows:

practically relevant, b) monitoring of microstructures, c) assessment of the reduced preophtal within constructively synthesized cortex and coat, d) high concentration of solutes with corresponding reduction in water content of the preophtal, e) reinforcement of lowered water content by pressures developed in the cortex, f) development of a solid support system of collagen dipicolinate with specific interactions of SPACOPs with sulfuric acids and possibly proteins, and g) construction of various layer coatings.

Goldman et al., (1988) showed that development of independent layers anterior to the cortex has little influence on host resistance of lenticles implantation. Using four morphotypes of apical tissue, host resistance did not correlate with apical water content, wet density, refractive index, or dipicolinate or cation content, but it did correlate with the volume ratio of preophtal to preophtal plus cortex. Bennett and Wertz (1988) determined collagen, hyaluronic, chondroitin-sulfate-dipicolinic acid (CDP), dipicolinic acid and hyaluronic in 32 spine preparations of lenticles implantation, with a 100-fold range in host resistance, and correlated their relation to host resistance. Collagen showed a small but significant increase with host resistance, while dipicolinate acid did not increase significantly. The best multiple regression equation for predicting host resistance included CDP, collagen dipicolinate and the hy-dic ratio.

(p=0.001). North (1985) proposed that spore dehydration is a critical, general mechanism for stabilisation of spore proteins to heat. As he of it is required to stabilise a spore enzyme in vitro to the same extent found in vivo. Removal of calcium dipicolinate and other low molecular weight spore substances did not have a major effect, thus supporting that dehydration of the core is achieved by water loss in the cortical layers.

Barrett (1981) reported an X-ray diffraction studies of bacterial spores and concluded that the DNA-DNA-DNA interaction and reduction of phosphate is right in the core mechanism of spore heat resistance. Lindsay and Barrett (1985, 1986) studied the effect of dipicolinic acid and its calcium salt on the spectral characteristics and the wet and dry densities of DNA. They concluded that interaction of dipicolinic acid with DNA influences the spore wet density and the ratio of core/core-cortex volume, and in way influence spore heat resistance. Dipicolinic acid was found to displace ethidium bromide from DNA, indicating that one mechanism of binding to intercalation (Lindsay and Barrett, 1986). This hydrophobic interaction would depend a nonfluorescently water could be displaced from spore polysaccharides, improving their resistance to denaturation. Randall et al., (1981) investigated the binding of ethidium bromide, and showed strong binding to spores which reduced the heat resistance of the spores after exposure to ethidium bromide.

Goodier (1931) studied the organization of DNA within surviving cells of Agellia species and showed that some species may contain more than one genome in their spore (Jain 1961). Zylberstein and Kohnenow (1972) reported the existence of a diploidic acid-fast mutant of M. goodii species that completely retained heat resistance, other Mycobacterium mutants retained only a fraction or completely lost heat resistance.

Ward et al., (1981) reported on the distribution of calcium and other elements in M. goodii. Almost all the sodium, magnesium, phosphorus and manganese were located in the core region, and a high concentration of silicon was found in the cutaneous layer. Foster and Brown (1981) studied spore-specific mineralization. They concluded that it is an important factor in spore heat resistance, but the relationship between resistance and mineralization is complex and dependent on initial temperature. Brown and Bennett (1981) studied the relationship among heat resistance and protoplast dehydration, mineralization and thermal adaptation. They found that increases in mineralization and thermal adaptation reduced protoplast water content between levels of ca. 52 and 10% (wet weight basis) and correlated with increases in thermal resistance. Above and below these levels, increases in mineralization and thermal adaptation correlated with increases in spore resistance independent of unchanged protoplast water content. The authors

suggested a molecular mechanism by which spores might stabilize spores by replacement of water by triglyceride, coating as well as the intercalation of DMG by calcium dipicolinate.

The influence of water activity on the heat resistance of bacteria was reviewed by Barrett and Slatt (1988), Gerry (1975), Jones et al., (1981), Barrett (1981), and Lindsay et al., (1988). Spore heat resistance as a function of its residues is maximum between 1.7 and 2.4. The effect of dried solids was also discussed. The influence of pH, its acid recovery temperature on *S. aureus* spore heat resistance of 150 different strains was explored by Brinkshulte and Parize (1971). Between pH 4.4 and 5.0, more heat treatments were needed to inactivate spores, soluble salts require relatively mild processes are sufficient. Jones and Barham (1988) investigated the correlation of spore heat resistance with proteolysis, autolysis, mineralization and thermal degradation. All three factors contributed to and were necessary for spore heat resistance, but hydrolysis predominated. Jones et al., (1981) investigated the correlation of heat resistance with water content, wet density and proteolysis/spore/hot volume ratio. An exponential increase in heat resistance correlated directly with wet density and inversely with water content and proteolysis/spore/hot volume ratio. Ryle (1981) also showed a correlation between heat resistance and the average decrease in proteolysis volume during sporulation.

The heat resistance of *C. botulinum* near the Δ range 0 to 2.5 was reported by Alvarado et al. [1982]. The equation reached was $\log_{10} \Delta = 0.4$. The correlation between proteolysis and proteolysis water content was reported by Lindsay et al. [1981] as a method for obtaining the proteolysis water content from the sugar density in *Strombosia* products. The average proteolysis dry density was 1.440 g/ml. The protective effect of fat on spore heat resistance was investigated by Senapati and Lentin [1977], Senapati [1977], and Abudado et al. [2004]. The relation to Δ was very important. The last authors also proposed a theory of spore clumping to explain the shape of experimental curves obtained as one possible explanation.

Activation

Activation is a process that changes viable spores incapable of germinating to an excruciating medium into activated spores capable of germination and growth. The usual method to activate spores is exposure to heat. Activation occurs for Δ in the range 2.0 to 2.5 [Kaplan et al. 1945]. Activation was realized by Kaplan et al. [1945], they mention that activation was first discovered in cold spores, that it was used as early as 1910 by Lentin, and that the main agents of activation are exposure to heat, to low pH (2-3.5), lipid compounds and strong oxidizing

spores. Their discussion of possible activation mechanisms supported the idea that the macromolecular components for resistance of the dormant stage are proteins rich in cysteine and stabilized by S-S linkages. Bentley (1977) said that the activation process was related to changes in tertiary structure of a protein responsible for the resistance of dormancy.

Freese and Gasterl (1965) found that spores of B. subtilis can be activated by exposure to calcium dipicolinate. Levinson and Ajello (1968) investigated activation of B. anthracis spores and found that they can be activated for germination on glucose by heating to an optimum temperature (not over 110°C), by treating with aqueous alkali adjusted at 10 deg-C, or by exposure to water vapor at room temperature. They postulated that activation involves hydration of a critical spore site, probably enzymatic, and that heating and alkali adjusted modify the molecular or physical properties of liquid water so as to resemble water vapor in its ability to reach or react with this site.

Levy et al., (1981) published an important paper on dormancy and activation of bacterial spores in which they reported that the kinetics of heat activation and deactivation do not conform to a pattern corresponding to two consecutive first-order reactions. First, calcium-streptococcus spores lost heat resistance but lapsed to when equilibrated with calcium. Spore equilibrium is facilis subitilis, including some thermodynamic properties of

The system, was thoroughly investigated by Davis and Zurel (1961). Forster (1961) found that spores of thermophilic bacilli could be activated by shifting them to and holding them at temperatures below their optimum growth temperature of 55 deg. C. The optimum reduced temperature for activation was 30 deg. C. Spores of B. pasteurianus failed activation at 30 deg. C were investigated by Forster (1965) who found that sodium nitrite was a potent activator. Complete spore activation was obtained after six hours or less at 30 deg. C. Sodium nitrate was ineffective as an activator at 44 deg. C.

Lesions

Injured bacterial spores are characterized by an inability, as a result of sublethal damage, to develop various stages of growth using standard growth media and techniques or even utilized for growth by untreated spores. However, they retain colony-forming capabilities when medium or growth conditions are modified. Modifications of growth requirements like reduction temperature, nutrients, germinants and increased susceptibility to inhibitors are signs of spore injury (Adams, 1954; Bock, 1958). Germination is a self-healing process, possibly with several pathways for completion of each stage. Complete, irreversible inactivation of one or more stages would trap a spore probably within the structures maintaining

domancy, and the spore effectively would be dead. If this inactivation can be observed, the spore is described as "injured".

Many of the manifestations of injury fall into four major classes: a) requirements by survivors for specialized particular stimuli, b) modified optimum incubation temperatures, c) increased sensitivity of survivors to inhibitors, and d) altered nutritional requirements of survivors (Jones, 1954). A general review of documented cases of bacterial spore injury was published by Pasquini and Barta (1961). Heat, irradiation, alkylate salts, acid, and other treatments were discussed together with the mechanisms: a) cellular damage, b) DNA damage, c) damaged or altered production systems, and d) damage to dispersion mechanism. A review of factors important to the detection of injured bacterial spores in processed foods was published by Johnson and Barta (1964).

The case of hurdling reducing spore injury has been investigated by several authors. Edwards et al. (1959) studied injury at different temperatures. Spores subjected to a hurdle temperature may be injured but not completely inactivated; consequently, heat injury may be an important factor in the evaluation of a successful sterilization process. These criteria indicated injury: a) significant reduction in survivors when spores were enumerated with a standard medium, but not when the standard medium contained added lactic and acetic dipicolinate,

b) more survivors of a changing host environment were enumerated with the standard method after incubation at 30 deg. C than at 45 deg. C (opposite to results obtained with untreated or slightly heated spores), and c) apparent number of survivors increased during an initial period of storage at 3 deg. C when enumerated with the standard method at 45 deg. C, indicating reversible spore injury. Ames and Barts (1974) presented results on host injury in B. subtilis as the inactivation of a spore germination system. The thermodynamic properties determined suggested that the mechanism of injury in this case was protein denaturation.

Smith (1964) and Smith (1966) reviewed repair in terms of existing knowledge on injury. Johnson and Jackson (1966) analyzed induction of DNA repair. The two principal pathways for repairing damaged DNA were a) direct reversal of an altered component back to its normal structure, and b) excision of defective material along with adjoining nucleotides followed by insertion of a normal nucleotide sequence utilizing an undamaged complementary DNA strand for base-pairing base reactions. Host induced damage to bacterial genomes and its repair was the subject of a review by Fritter and Slonay (1964).

Delayed germination of heat damaged B. subtilis spores was studied by Lysa et al. (1963). In that paper, any culture requiring more than one week to show evidence of growth was considered delayed. Incubation was prolonged up to 12 weeks

The value of thermal reaction time is increased as much as fourfold, and proteolytic enzymes showed substantial increases in t values. The recovery of *Escherichia coli* after irradiation was investigated by Dapkin et al., (1966). These factors were shown to affect recovery of the biological indicators: a) incubation temperature, b) post-sterilization hold time, and c) post-sterilization storage temperature. These factors should be adequately controlled in order to obtain reproducible results.

System Analysis of Population Dynamics

System analysis is a body of concepts and methods for analyzing complex systems. Its development and applications continuously expand. It is an attempt to establish knowledge and understanding, and its application to the fields of food engineering and microbiology will facilitate the solution of complex, practical problems. The last section of this review is a brief examination of the most important concepts of system analysis pertinent to biological systems. The main reference used for this section is Savage (1966).

Because of the interdisciplinary character of system analysis there are necessarily numerous definitions. A system is a composition of objects which comprise a relative, functional whole exhibiting characteristic behavioral properties. To be a

concepts, a system must consist of a) a set of objects and b) a set of interconnections. External stimuli received by a system are called inputs, and the responses of the system are called outputs. Within a system circulate attributes, called state variables, measure the internal condition of or state of the system. Behavioral analysis examines causal relationships between inputs, state and output variables to determine and characterize behavioral properties of a system.

Anatomical and physiological systems are amenable to the same representational theory as physical systems when analyzed from the process-oriented approach followed here. Four genres of process—storage, transport, transformation and source of risk—and their generic descriptions are adequate to describe most, if not all, types of systems.

Diagrams are commonly used to represent and to support verbal descriptions of systems. Only three elements of the graphical system employed in this research need to be defined here; these elements are depicted in Figure 4. A score, depicted by the object in Figure 4a, represents a population. The object in Figure 4b represents a transformation such as metamorphosis or mortality, of individuals between specified populations. A sink is a population that is no longer able to participate in the process of a system; it is depicted by the object in Figure 4c.

Each object in Figure 4 has one or more extensions called terminals. Terminals provide the graphical means for

Figure 4 Representation of system components: store (q), transformation (ϕ), and sink (α).



interconnecting component processes composing a system and for representing resulting interactions. Associated with each formal and aggregate variables denoting extensive and intensive attributes relevant to the description of the object. The process-oriented approach to modeling employed here identifies the extensive variables of a component with the flow of individuals within to a component conjugate to the intensive property associated with the interest.

A population is a distinct group of organisms with common type. Typically the size of a population fluctuates with time in response to natality, development, mortality and migration. Population characteristics do not include details about individual members, but attribute focuses on properties of the aggregate. Extensive properties, such as number of individuals, space occupied, and flows of individuals depend on the size, or extent, and content of a system component. When two elements of a species are aggregated, extensive parameters of the composite equal sum of corresponding parameters of the individual states. Intensive properties, such as temperature, concentration and population density, do not depend on the size or extent of the component. A principal population sink is that for the dead, and in this work, inactivated agents are stored in this sink.

In general, the diagrams, called process diagrams, describe ecological processes, their manner of interconnection and important system variables. Analysis of component functions identify interfaces between the components involved. Interfaces are sites of component interactions, and they are indicated in diagrams by dots at junctions, which are called vertices, nodes, or interfaces.

There are several examples of biological transformations: metamorphosis of insects and transformations between distinct living states, activation, inhibition and injury of bacterial species, etc. Fecundity transforms individuals from a live state to a dead state regardless of the cause (physiological failure, disease, predation, parasitism etc.). Development of an organism is a complex physiological and morphogenetic process where age classes may be considered, together with the physiological rates of development (aging). Reproduction is the key to propagation and maintenance of a species. Reproduction is a transformation that connects the adult population with the first age-class population. Fecundity largely depends on environmental factors, and that dependence may be accounted for among related functions in the transformation matrix. A transformation representing mortality removes members from a state of live individuals and transfers them to the population sink. Predation and parasitism are distinct forms of mortality, but their representation and discussion is similar: a transformation

representing predation (parasitism) expresses the causal dependence of prey (host) size on prey (host) density and predator (parasitoid) density; thresholds of the transformation connect the affected state of prey (host) species to a risk for the death and replicate the state of relevant predatory (parasitoid) species.

Models are operational statements of knowledge and their application strengthens different fields of knowledge. Models of populational systems are developed by logically and systematically performing four steps:

1. Develop a conceptual model by decomposing a given real system into a network of perceived populational components.
2. Experimentally and/or theoretically determine the description of each component in the conceptual model.
3. Formulate the structural description of the system, as depicted in the conceptual model.
4. Derive a mathematical model corresponding to the conceptual model and descriptions of components and structures. It will consist of one or more equations representative of the whole system as an entity.

Generic descriptions of the four fundamental families of processes guide experimental and theoretical descriptions of specific real processes. The level of system structure facilitates mathematical description of that aspect of a system (Senge,

1999). Several methods, including state variables, circuit and node formulations, exist for formulating a mathematical model of a system (Koenig et al., 1983; Martins and Allen, 1999).

RESULTS AND METHODS

Scope of Work

The scope of work undertaken in this study has been divided into two parts addressing the two most important factors affecting reliability of commercial sterilization processes with irradiation units (24,25). These factors are the heat transfer characteristics of the unit and the inactivation kinetics of the indicator microorganisms.

A series of experiments was performed to determine the contributions of heat transfer and material strength characteristics of RIGs to the discrepancy between predictions by higher a kinetic model and corresponding experimental data. In these experiments, various L. monocytogenes strains from different heat treatments were measured and compared to corresponding expected values calculated using traditional kinetic models with the time-temperature history of the treatments. Later, RIGs were designed and built from aluminum for improved heat transfer and strength characteristics, and their performance was compared to that of plastic RIGs normally used in commercial practice.

Finally, the kinetics of bacterial spore inactivation was studied in four steps. In step one, a new kinetic model was developed of a suspension of bacterial spores treated at initial

temperatures. The ecological model was proposed first from an application of the theory of population dynamics, which led to development of a mathematical model as a set of differential equations describing the various reactions (transformations) involved. The solution of these differential equations produced response equations that consisted of sums of exponential terms.

The next step in this part of the work was determination of parameters of the model, as consisted of laboratory experiments involving carefully controlled time-temperature exposures of *B. subtilis* spore suspensions. Data from these experiments were used to produce experimentally-derived survivor curves for both normal and injured spores. These curves were analyzed to determine values of parameters of the model, which were coefficients and arguments of exponential terms in the response equations. Subsequently, predicted survivor curves were compared with experimental survivor curves at each tested temperature used in the thermal exposure experiments.

In the third step, temperature dependence of the first-order reaction rate constants was determined in accordance with the Arrhenius equation, and it substituted for the rate constants in the new kinetic model. In this form, the model should be capable of predicting the number of survivors of a thermal process in which the lethal temperature was varied as an arbitrary function of time (a transient temperature process).

The fourth step was a final test or validation of the model in which experimental results of a transient temperature process were compared with results predicted by the model for the same temperature history.

Details of methodology are presented in the following subsections, beginning with a description of how spore suspensions were prepared and handled throughout the study. Then, the methodology used in carrying out the heat transfer experiments with *Microcococcus* cells is discussed. Finally, development and testing of the new kinetic model are discussed.

Theory formulates provides the rationale for the methodology described in the following sections, identifying significant variables, their ranges of variation and the corresponding practical aspects. Hence, theory formulates led to a typically structured experimental design. It is presented for the study of both heat transfer in stills and kinetics of bacterial spore inactivation in terms of available information gathered from the literature review to support various assumptions and reported, logical references.

Spore Suspensions and Formulation of Surrogates

Bacillus stearothermophilus spores. The spore suspensions used were provided by two laboratories, already placed under BSL-2. The thermal resistance parameters were independently

determined as $\text{OD}_{550} = 0.73$ and 2×10^8 cells ml^{-1} by an outside laboratory at the University of Saskatchewan (Eaton, 1984). The suspension was kept at 4 deg. C.

Isolation, growth, and storage of "A" species The suspension used was more than three years old, and was obtained from Prof. F.J. Davis at the University of Florida Food Institute and Marine Fisheries Experiment, washed and stored at 4 deg. C. Inspection under the phase contrast microscope showed the spores to be fully refractile and free from vegetative cells, phase dark spheres and clumps. The direct microscopic count was performed in a phase contrast microscope using a bright-line haemocytometer (American Optical).

Isolation of bacterial species For the isolation of *B. subtilis* (ATCC 6051) from our spores, the growth medium was Spectrum soy agar, provided by Ross Laboratories. The medium was prepared by adding 20 g. to 500 ml of distilled water, and pH was adjusted to 7.2. Then the medium was placed in four flasks and stored for 30 min and stirred and quantified at 200 deg. F (121 deg. C) for 30 min. Decant dilutions of the suspension (100 μ l) were prepared in sterile distilled water, and 1 ml of each dilution was placed in a Petri plate with ca. 30 ml of growth medium added. The incubation temperature was 30 deg. C for 48 hr.

The growth medium used for *B. subtilis* was that described by Edwards et al. (1984), in the case when an calcium dipicolinate

was added. Two incubation temperatures were used: 35 deg. C for 14 hrs. (standard incubation temperature for this species), and 20 deg. C for 14 hrs. (for detection of injured spores). Study of the type of spore injury occurred in this work is dependent upon simultaneous or independent activation of a germination response that allows spores to grow at lower temperatures (Gowans, 1943).

A brief tube dilution (Porter-Gordon) was used to alter dilution tubes and plates for 15 s. In all cases, decimal dilutions of the experimental samples were prepared in sterile distilled water, and 1 ml of each dilution was placed in a Petri plate with or 20 ml of growth medium added. In the case of *S. putrefaciens*, two plates were prepared of each dilution, corresponding to the two incubation temperatures used. When the medium solidified the plates were incubated, and the resulting colonies were counted.

Investigator's Will

In this section, procedures corresponding to the study of Will are presented. Propositions, assumptions and logical inferences that led to the experimental design are presented first.

Propositions

1. The temperature history of a biobiosensor unit should be as close as possible to that of the cold spot of the product (Joshi, 1993; Smith, 1999).
2. Pressure changes can affect the lethality (Palative et al., 2001; Dinkley and Gertlerkamp 2001).
3. The time-temperature history can be measured using microencapsulated thermocouples.
4. The number of survivor spores can be measured microbiologically (Joffe et al., 1993).

Conclusions

1. The conductivity of the material used to fabricate a hot plug plays an important role in its ability to accurately transmit the temperature history of the product undergoing thermal treatment.
2. The mechanical strength of a thermosensor wall is important when pressure changes occur during a sterilization process.
3. The effect of container material on the performance of a biobiosensor wall can be studied using heat-penetration measurements and microbiological assessments of survivor counts.

Acknowledgements

Improved performance of biobiosensor units can be achieved when fabricated from materials with high thermal conductivity

and mechanical strength (i.e., metal vs. plastic) and this improvement is more important for rigid semi-rigidified liquid products than for slow solidification products.

Experimental Work

Following the guidelines established in theory formation, plastic films were tested using both constant and variable temperature processes. Next, plastic films were compared to aluminum films in a constant temperature process. The experiments with plastic films were designed to test the traditional kinetic model under different process conditions, while the comparative experiments using plastic and aluminum films at constant initial temperatures were designed to test the relative importance of heat transfer to the selection of desired solidification processes using film. The higher thermal conductivity of aluminum should allow the internal temperature of an aluminum film to follow the product temperature more closely and, thereby eliminating inaccuracies related to heat transfer.

Instrumentation. The plastic films (PVC or PE, 1968) were provided by Rex Laboratories (Lafayette, IN). The aluminum films were made with the same dimensions as the plastic films, using a two-part construction in which the top portion holding the upper suspension was made of aluminum, while the remaining

support section was made of plastic to keep the aluminum section well insulated from the conical-rod interface. Two lifts were constructed with needle thermocouples and saturated distilled water. A photograph of the lifts is presented in Figure 1.

Procedure. The heat treatments for experiments with plastic and aluminum lifts were performed using a vertical steel cast reheat. Figure 2 shows a 40-mm-dia (2.17-in.) 1040 steel reheat exposed with copper-constantan needle thermocouples (0.1° F) behind custom thermocouples (Type 6041, Fluke) and lifts. The different probes were placed at approximately the same position inside the cast. The time-temperature data were recorded on a Digiscan II data logger (Rye Instruments, Bedford, Mass.) connected to a 80-2 computer system (Digital equipment corporation, Ryeport, Mass.). The cast prepared with lifts and thermocouples were filled with hot distilled water until there was a 0.2 in. headspace, then they were sealed and placed inside the reheat. The thermocouples were connected to the temperature recorder, and the reheat was closed. After the selected soaking time of 2 min, the soaking valve was closed, and the temperature was regulated to follow the prescribed thermal history. When the process time was reached, the steam valve was closed, the pressurized air valve was opened, and cooling water was allowed to enter the reheat. Afterwards, the reheat was opened, and the lifts were recovered from the cast. The lifts were immediately placed in ice and stored under refrigeration.

Figure 7: Plots of the aluminum microheater series, and of their instrumentation with a single thermocouple



Figure 1 The (4) samples analyzed with a copper
catalyst under acidic conditions and
plastic and aluminum substrates
in 12.



Reliability was calculated by numerical integration of the following standard expression:

$$R = \int_0^T \frac{(T-298)^2}{10} dt \quad \text{eq. (14)}$$

where R is the reliability, T is the temperature at each time interval adopted in eq. (14), T_0 is time in min, and 1 is the temperature dependency factor for thermal resistance. Temperature measured for a test-PdM change is added into Temperature history data from the first treatments were used in these integrations. The calculated reliability was compared with the reliability found by microbiological observation of the carrier spores, which can be expressed as R_{spores} .

$$R = R_{\text{spores}} \log(N_0/N), \quad \text{eq. (15)}$$

where N and N_0 are final and initial values of carrier spores respectively and R_{spores} is the decimal reduction time for the spore suspension at the reference temperature.

During the course of this work, it became apparent that differences in mechanical strength between the plastic and elastomer might be a factor in explaining observed behavior. Materials testing was performed to study high temperature softening of plastic ADPs.¹² Force-deformation curves for the plastic ADPs at high temperatures were determined using a 20

Rotational Test System (Rohden Instruments) equipped with an oscillographic 3000 recorder. Fluctuations of the environmentally controlled chamber did not perturb experiments at higher temperatures. The samples were hollow cylinders prepared from the plastic B18 with 1.763 in. (0.04493 in) outside diameter, 1.426 in. (0.036176 in) internal diameter, and 0.836 in. (0.021177 in) height. The speed was 0.0033 rad/s. Ten tests were performed: one at 20 deg. C and another at 50 deg. C.

The plastic B18 were subjected to two thermal regimes: a constant robot-temperature of 200 deg. F (128.3 deg. C) and a variable temperature wherein the robot temperature was raised first to 200 deg. F (128.3 deg. C), and then to 300 deg. F (154.47 deg. C). The type of process was initially considered as an important factor in explaining the divergences between initially found by integration using Poplaw's model and initially found by measurement of curves. The experiments to compare the performance of elastomer with plastic B18 were performed at a constant temperature of 200 deg. F (128.3 deg. C); therefore, the first set of experiments attempted to test the effect of different types of heat-treatment on the performance of plastic B18, while the second set tested the importance of the heat conductivity and mechanical strength on the performance of the different B18.

Outline of agent modelization

In this section, the propositions, assumptions and logical inferences in the theory formation and the system(s) development of a model of a suspension of bacterial agents are presented. The experiments performed for parameter determination and validation of the model are described.

Propositions

1. Activation, inactivation, and injury are observable variables that occur during host treatment of bacterial agents [Kata, 1979; Kata and Greif, 1984; Edwards, et al., 1984].

2. System analysis is a set of techniques that can be applied to the study of population dynamics with multiple concurrent reactions [Gause, 1983].

3. The number of survivor agents can be measured accurately/qualitatively [Kata, 1983].

4. The temperature history of host treatments can be precisely measured and controlled [Kata et al., 1984].

Assumptions

1. The transformation can be described by simple first-order reaction kinetic models.

2. The transformations can occur simultaneously
3. The transformations are independent of each other
4. The dependence of each rate constant on temperature can be described by an Arrhenius-like model

Abstract

The effect of initial temperatures on bacterial spores can be modeled using systems analysis of population dynamics, if the microbiological observation of the survivor spores and the measurement of the time-temperature history can be accurately obtained for a given spore suspension.

Summary of Research Work

In this phase of work, and in accordance with theory. Namias, the kinetics of spore survival at initial temperatures were studied as a major factor in the biological variation of thermal sterilization processes. The main changes (biotransformations) that occur during exposure were identified from a review of the literature and incorporated in a conceptual model amenable to systematic analysis. A mathematical model was generated and a series of experiments at different constant temperatures were performed. These experiments provided data for estimation of parameters in the model, including Arrhenius

description of the temperature dependence of rate constants. As an initial validation of the model, the results of a nonisothermal experiment were compared with predictions of the model for the temperature history recorded during the experiment.

Model Development

A conceptual model of a suspension of bacterial spores at lethal temperatures was proposed, and components and interactions were identified. The components of the system are the different stages of a population of bacterial spores undergoing a heat treatment at lethal temperatures: inactive nonactivated spores, activated spores, injured spores, and a less heat resistant population. The transitions among these subpopulations are multiplication (λ), activation (α) and injury (β). Rates, representing the populations, and transformations were analyzed together with their possible interactions. Mathematical expressions describing the transformations were then incorporated, and a system of differential equations in the subpopulations was generated. Solutions of this system of differential equations for the full thermal cycle, taking into consideration the initial conditions, allowed prediction of the populations at different moments.

Conceptual model. The assessment procedure is very important to the definition of components in the system. Spores are considered active if they are able to grow when incubated at 40 deg. C. They are considered injured if they are capable of growth when incubated at 37 deg. C, but not at 40 deg. C. They are considered inactivated if they no longer are able to germinate and grow when incubated at either temperature. A less than resistant population consists of quantified spores that have lost their heat-resistance or vegetative cells or, in the case of multiple phase bacteria, spores that are genetically less heat-resistant. However, a less heat-resistant population can produce colonies in an enrichment medium that are indistinguishable from colonies originated by active activated spores of the system.

The conceptual model is presented diagrammatically in Figure 1. Active spores that have not been activated (dormant) are potentially able to produce colonies after activation, they comprise the population in state R_1 . Activated spores, potentially capable of producing bacterial colonies in the enrichment medium, are represented by state R_2 . Injured spores are determined by their ability to grow at relatively low incubation temperature (37 deg. C). They are denoted by the additional subscript 1. An injured population has analogous subpopulations of active deactivated and activated spores, R_{11} and R_{12} respectively. In this case, the germination restriction allows

Figure 3 Spine diagram for a fluidized spent suspension under treatment at lethal temperatures. The subscript 2 refers to a lean heat recycled fraction, the subscript 1 refers to viable normal spores, the subscript 3 refers to activated spores, and the subscript 4 refers to an alternative generation mechanism or to spore injury



Activation of alternative germination
mechanism or injury



activated spores to grow at lower incubation temperatures... The equivalence of less heat-resistant microorganisms that may be present are represented by strains H_{90} and H_{10} for normal and injured classes respectively. Another equivalent of possible ignorance, but not included in this work, is the fraction of spores that may survive during the heat interval, thereby increasing the number of spores to H_1 or H_2 . This transformation would lead to dispersion of the values obtained for the number of survivors. It should be minimized by using a homogeneous spore suspension.

The hypotheses that the transformations are simultaneous and independent were very important to the conceptual model; it has four major independent parts. The hypothesis of consecutive reactions has been previously studied and found incorrect (Lark et al., 1983), and no reason could be found to justify further coupling of the reactions. The upper left part of Figure 3 represents inactivation of a less heat-resistant fraction H_{10} . The upper right part represents collection (q_1) of natural viable dormant spores (H_1) and inactivation of both populations in agreement with the hypotheses that these transformations are simultaneous and independent. The lower part of the diagram represents injury-related events in an analogous manner; the left part represents the inactivation of a less heat-resistant population (H_2) that could be inactivated using a lower incubation temperature (121 deg.C), the right part represents the

injury of viable spores, together with the inactivation of sub-populations (R_1, R_2). This type of injury may be described as the activation of a germination mechanism that operates at lower temperatures than the normal germination mechanism. The triangles represent the size for the inactivated spores.

Sublethal injury. Since the four parts of the model are not coupled they can be analyzed independently. The components are described as follows

Sizes

$$R_1 = -\frac{dR_1}{dt} = -\frac{1}{1} \times \text{inactivation of } R_1 \quad (14)$$

$$R_2 = -\frac{dR_2}{dt} = -\frac{1}{2} \times \text{inactivation of } R_2 \quad (15)$$

Transformations

Activation

$$R_2 = k_1 R_1 \times \text{inactivation of } R_1 \times \text{inactivation of } R_2 \quad (16)$$

Inactivation

$$T_{12} = k_2 R_2, \quad (k = 1, 2) \times \text{inactivation of } R_1 \times \text{inactivation of } R_2 \quad (17)$$

Inactivation of heat-resistant fraction

$$T_{10} = k_3 R_1 \times \text{inactivation of } R_1 \times \text{inactivation of } R_2 \quad (18)$$

A generally no flow between stores is level mathematically on the law of conservation of matter: since the nodes cannot act as a store, the quantity of flow at every node is zero. Consequently, the structure of the system is described by the following equations:

Summation of flow at node a:

$$- \dot{V}_1 + \dot{V}_{1a} + \dot{V}_{1b} = 0 \quad \text{or} \quad \dot{V}_1 = + (\dot{V}_{1a} + \dot{V}_{1b}) \quad (11)$$

Summation of flow at node b:

$$\dot{V}_{1a} + \dot{V}_2 + \dot{V}_{2a} = 0 \quad \text{or} \quad \dot{V}_2 = -\dot{V}_{1a} - \dot{V}_{2a} \quad (12)$$

Substitution of the equations for the transformations (11, 12, 34) in Equations 25 and 22 yields

$$\dot{V}_1 = + (K_1 Q_1 + K_2 Q_2) = + (K_1 + K_2) Q_1 \quad (13)$$

$$\dot{V}_2 = K_1 Q_1 + K_2 Q_2 = - (K_1 + K_2) Q_1 \quad (14)$$

The mathematical model corresponding to this part of the conceptual model is obtained by substituting Equations 13 and 14 in the equations for the stores (26 and 27). It is presented with the initial conditions used in this research:

$$\frac{dQ_1}{dt} = + (K_1 + K_2) Q_1, \quad Q_1(0) = Q_{10} \quad (15)$$

$$\frac{dQ_2}{dt} = K_1 Q_1 + K_2 Q_2, \quad Q_2(0) = Q_{20} \quad (16)$$

Solution of the model with the initial conditions yields the response equations

$$R_1(t) = R_{1app}[-(R_1 + R_1(t)) \exp(-R_1 t)] \quad (17)$$

$$R_2(t) = [R_{22}R_{22}(max)-R_2(t)-R_{2app}]{-(R_2 + R_2(t))} \quad (18)$$

By similar means, a single differential equation is derived for the less fast-reactant fraction, together with an initial condition

$$\frac{dR_2}{dt} = R_2R_{22}, \quad R_2(0) = R_{2p} \quad (19)$$

The solution is

$$R_2 = R_{2app}[-R_2(t) \exp(-R_2 t)] \quad (20)$$

The case of rapid species or activation of an alternate generation mechanism are handled similarly. The mathematical model is presented in Table 1, and the corresponding equations for the responses are presented in Table 2.

Table 1. Rationalized model of a suspension of bacterial spores at fixed temperature.

$\frac{dN_1}{dt} = - (D_1 + k_1) N_1$	$N_1(0) = N_{10}$
$\frac{dN_2}{dt} = k_1 N_1 - D_2 N_2$	$N_2(0) = N_{20}$
$\frac{dN_3}{dt} = k_2 p N_2$	$N_3(0) = N_{30}$
$\frac{dN_{11}}{dt} = - (D_{11} + k_{11}) N_{11}$	$N_{11}(0) = N_{110}$
$\frac{dN_{12}}{dt} = k_{11} N_{11} - D_{12} N_{12}$	$N_{12}(0) = N_{120}$
$\frac{dN_{13}}{dt} = k_{12} p N_{12}$	$N_{13}(0) = N_{130}$

In consequence, the form of the equation for the survivors (N) is a sum of three decaying exponential terms, one of which is negative,

$$\dot{N}(t) = (N_0 - N_{\text{agg}}) \lambda_{\text{agg}}(-\delta t) - k_{\text{agg}} N_{\text{agg}} - (\delta t + \delta_0(t)) \phi_{\text{agg}} N_{\text{agg}} - \delta t(t_1, \dots, t_2)$$

The term that accounts for the loss heat-resistant fraction produces an initial drop in the number of survivors. The other positive term pertains to the traditional single exponential describing the preheated population. This exponential term takes longer to decay than the other two, and it becomes dominant after an initial period. The activation part of the survivor curve is characterized by an initial rise, followed by a transition zone (sometimes referred to as "shoulder") and later by a decrease in the number of survivor spores (see Figure 4). It is determined by the combination of the two terms that account for the activated spores. The negative exponential explains the initial rise in the number of survivors. Therefore, the mathematical model should be able to describe changes with time in the number of survivor spores under thermal treatment, and the physical meaning of the exponential terms in the response equation is clear.

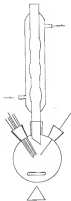
Experimental Apparatus

The experimental apparatus permitted experiments for parameter determination and validation of the model. It consisted of a three-neck flask equipped with a magnetic stirrer, a reflux condenser and a set of 3 needles used to inject an aliquot of the spore suspension and to retrieve samples, as depicted in Figure 26. The thermometer was connected to an analog-to-digital (A/D) interface by means of an amplifier board. The A/D interface, in turn, was connected to an IBM-PC compatible microcomputer and to a heater placed under the three-neck flask. This computer control system permitted accurate control of the temperature of the dilute spore suspension inside the flask. The temperature was continuously mixed with a magnetic stirrer, while time-temperature data were recorded in a computer file. A condenser placed in the middle neck received deionized water and maintained a constant volume of water in the flask.

Procedure

The flask, the septa, needles, condenser, thermometer, 50 ml of distilled water and a syringe for injecting the spore suspension were sterilized before each experiment. Sterile, disposable syringes were used for sampling. Special care was

Figure 18. (Experimental) apparatus: Three-neck flask equipped with a needle thermometer, a reflux condenser and a set of five sampling needles



taken to assure mixing of the sperm suspension by using a vortex mixer for at least 20 s before taking each sample.

The opportunity was allowed to reach equilibrium at the desired incubation temperature, and then a 1.0 ml aliquot of sperm suspension at room temperature was injected into the 10 ml of sterile, distilled water through one of the needles installed in the left neck of the flask. This changed the internal temperature of the flask less than 0.5, and the process temperature was regulated after a few seconds. An attempt was made to measure or regulate the pH (pH of distilled water is 8 - 8.3, the heat resistance of sperm at pH 7 is expected to be higher). Eight consecutive 0.5 ml samples were taken in each experiment, and the corresponding times were recorded. A different needle was used for each sample. Similar samples were taken of the untreated suspension as a control. The samples were immediately mixed with 0.5 ml of cold sterile water, and kept on ice or in a cold room with the microbiological enumeration of the survivors could be performed. To evaluate the dispersion of the data, four repetitions of one point were made in order to provide an estimate of the standard deviation used later in the nonlinear regression analysis.

To obtain initial, tentative, values for parameters in the model, as required for nonlinear regression, experimental data were analyzed by the method of successive residuals (Petersen, 1974) which is very useful for curve fitting when the model is a

sums of exponentials. The difference in the exponential time constants determines their relative rate of decay. After a sufficient interval the slowest exponential in the sum dominates, and it can be determined by regression. When this exponential term has been determined, the experimental values can be subtracted from it, leaving another exponential to dominate. It, too, can be determined by regression. This procedure is applied successively until the differences between the experimental values and the last exponential determined become negligible.

The procedure to find good initial values for the nonlinear regression is illustrated below for a two-exponential case (subinhibition-inactivation). The equation is

$$y = n_1 \exp(-k_1 t) + n_2 \exp(-k_2 t) \quad (11)$$

where y is the number of surviving spores at time t . The region where the positive exponential term constant is found by inspection of the survivor curve, and the values of n_1 and k_1 are determined by linear regression of the squared logarithm of y in that region. Since the positive exponential and the values of t are known, the negative exponential can be determined by subtraction

$$k_2 \exp(-k_2 t) = n_1 \exp(-k_1 t) - y \quad (12)$$

This second experiment is referred to as the first residual. The extent of successive residuals is very variable. To check this, dispersion of the experimental data in this study was too large for exclusive use of this method, but it was utilized to find good first estimates for use in the nonlinear regression procedure.

A computer program was developed for nonlinear regression analysis of the data using the Levenberg-Marquardt method, following procedures described by Press et al., (1988). The value of chi squared was used as the criterion of the optimization procedure that searched for a minimum, and convergence was considered to be achieved when the change in successive chi-squared was smaller than 10^{-6} . In order to reduce the statistical error (round off), data in the experiments was divided by 100, and experimental numbers of variables were divided by one million. This scaling of variables was required to facilitate use of the computer software and to improve its accuracy and stability.

Isothermal experiments were performed at 67, 70, 76, and 79 deg. C. Values obtained for the rate constants were fitted to an Arrhenius plot to determine the dependence of rate constants on temperature. The Arrhenius type formulas were compared with the response equations for constant nonisothermal cases. The nonisothermal experiment was similar to the isothermal experiment, except that the temperature was deliberately varied

with time by turning the heater on and off several times during a process while keeping an accurate record of the floor temperature history thus experienced.

In addition to the data generated from the experimental work, another set of data was used to test the model. These data for Chironomid larvae in soil were obtained from Dr. L. Aelbrecht at the Department of Microbiology, University of Wisconsin at the Institut Agronomique et Veterinaire Suisse II in Geneva.

The theoretical and mathematical parts of this research led to the design and execution of a series of experiments. The results obtained are presented and discussed in the next section.

RESULTS AND DISCUSSION

Experiments with Bifidobacter Cells

Experiments performed in the first part of this research used a suspension of spores of the thermophile Bacillus stearothermophilus as the bifidobacter analogue inside cylindrical bioreactor units (BUs). The BUs were made of plastic or aluminum. These experiments explored the importance of heat transfer to using BUs in the realization of canned sterilization processes. Results obtained are presented in the following section.

Heat Transfer

Time-temperature graphs shown in Figure 11 represent typical temperatures inside the BUs and the retort used in these experiments. There was a period in time when the temperature inside a plastic BU rose above the retort temperature. This happened when pressurized air was allowed to enter the retort at the beginning of the cooling phase. The rate of heating of plastic containers was always less than that of aluminum containers. The timing of BUs to follow the thermal history of the contents of a can is shown in Figures 12 and 13. 4

Figure 11: Time-temperature curves for the rubber, plastic and ceramic PZT/polymer cells

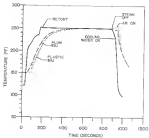


Figure 12. Heat penetration curves for the plastic
6Li and the contents of the tin using an
uncoated tungsten-tungsten form (left), (50) and
(Kondo, 1962)

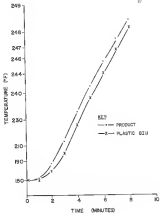
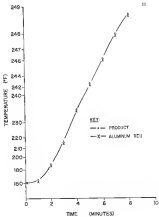


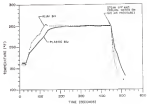
Figure 11: Heat penetration curves for the aluminum lid and the contents of the can using an improved one-layer finite difference (Hall, 1992 and Suenka, 1994)



comparison of temperatures inside a bioreactor with the corresponding point in a run is presented in the form of standard heat distribution curves. This presentation emphasizes the differences that may occur when the product temperature is near the referent temperature. Contributions to process lethality in this region are very important (Suenko, 1948, page 132). Figure 13 shows that the temperature experienced inside an aluminum BIA follows the external temperature history very closely (the curves are nearly identical). Likewise, at some points in time within the tested range, the temperature inside a plastic BIA appreciably lagsged the product's temperature. Likewise appeared to have no detrimental effect on the spore suspension, since plate counts following a given thermal process were of the same order of magnitude in both plastic and aluminum BIA. The plate count information is presented later.

The unexpected rise in internal temperature of a plastic unit coincided with the time at which high pressure air was admitted to the reactor to avoid expansion of the run just prior to sterilizing cooling water. In order to confirm this observation, additional experiments were conducted with the BIA exposed directly to the referent environment under various cooling cycles with and without over-riding air pressure. These experiments always showed a rise in temperature in a plastic bioreactor only when over-riding air pressure was admitted to the reactor. Figure 14 shows that the temperature in a plastic BIA did not

Figure 14: Selected trajectories of ϕ_{local} and algorithm 1126 when no perturbed α is selected from the history.



rise when pressurized air was not used. A rise in temperature was not observed with aluminum containers when over-riding air pressure was admitted. These results prompted an examination of the mechanical strength properties of plastic film.

Figure 13 presents the effect of temperature on the force-deformation curves for samples prepared from plastic film. The temperatures used were lower than typical temperatures in sterilization experiments because of limitations imposed by equipment. The peak values decreased from 2530 N at 70 deg. C to 2230 at 92 deg. C. When a container is ruptured by external pressure and its volume is reduced, the internal temperature should rise as is explained in the next section of this chapter.

Percent Leakability

The values for k at 150 deg. F (65.5 deg. C) and for k (92 deg. F) were determined independently for the two open temperatures at the University of Massachusetts (Dunn, 1984). The leakabilities were calculated for these experiments by numerical integration using the variable temperature history for the six fermentations. Leakabilities calculated from the microbiological inhibition of bacteria and from integration of the time-temperature data are summarized in Table 3.

Figure 15 Effect of the temperature on the force-deformation curves for samples prepared from plantain flour

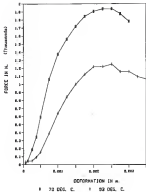


Table 1. Lethalities (avg) from several different treatments using plastic and aluminum insecticide units, and labeled Carbaryl insecticide as insecticide exposure

Case	Aluminum Insecticide	Plastic Insecticide
Two different processes using plastic film		
Self temperature process	29.3	18.3
Quatern temperature process	34.1	30.3
Comparison between plastic and aluminum film		
Plastic film	32.34	21.9
Aluminum film	37.08	26.4

The mortalities obtained by the two methods differ by about 10 mm, a significant disagreement. Similarity of the calculated lethalities for the aluminum and plastic film occurred because with plastic units the temperature lag during cooling compensated for the lag during heating in the traditional process used. Similar information on the number of survivors is presented in Table 4. The authors' procedures in Table 3 give very important discrepancies between two different methods that theoretically should give same results. The differences between replicates were smaller, around 15%, than the

differences between the two methods. In some cases, the differences were by several orders of magnitude. But smaller considerations also cannot explain the differences found.

Table 4. Surveys from several different treatments using plastic and aluminum indicator wells, and *Basillus thuringiensis* throughly to indicator organisms

Case	Physiological	Calculated
Two different processes using plastic film ^a		
24/24 temperature process	$4 \cdot 10^{10}$	$3 \cdot 10^{10}$
Control temperature process	$4 \cdot 10^{10}$	$3 \cdot 10^{10}$
Comparison between plastic and aluminum film ^{ab}		
Plastic film	$2 \cdot 10^{10}$	$3 \cdot 10^{10}$
Aluminum film	$2 \cdot 10^{10}$	$1 \cdot 10^{10}$

^a Initial number of viable spores $2 \cdot 10^8$

^{ab} Initial number of viable spores $2 \cdot 10^8$

Discussion. Because in this part of the study no separate experiments were designed in response to the results of previous work, this allowed subjective answers to

understanding the problem. Initially, some information was available from the literature and private industry on the discrepancies between final spore counts found using plastic film containing a *Bacillus pasteurii* spore suspension and the number of surviving spores determined by simulation with Ingber's model for the thermal history of a sterilization process. A set of experiments was performed to verify the accuracy of these reports, using constant and variable temperature processes. The discrepancies in survivor spore counts were by two orders of magnitude regardless of the type of thermal process involved (the upper part of Table 1). The performance of Ingber's model was not good enough for routine use in the control of thermal sterilization processes.

A second set of experiments at constant retort temperature was designed to test the influence of heat transfer on the performance of ALIs. Aluminum ALIs were designed, fabricated and tested against plastic ALIs. The experiments yielded time-temperature histories (Figures 11, 12 and 13). Figures 12 and 13 are presented in the form of standard industrial heat penetration curves (Moore, 1968), that emphasize the temperature region where the internal ALI temperature is near the retort temperature. This region is where a very high lethal effect is achieved. Inspection of the time-temperature data confirmed expectations that aluminum ALIs followed the thermal history of the contents of a can closely enough to eliminate

heat-transfer as a source of discrepancy in spore counts. In this set of experiments, spores were not heat-activated previous to their exposure, and a control plate was used before and after exposure to star the spore suspensions. The numbers of surviving spores in these experiments are presented in the lower part of Table 4. The discrepancies in spore counts are between one and two orders of magnitude, and they are far larger than the 30% variation being replicated. Inspection of thermal histories revealed an interval of pressure increases at the onset of cooling that caused the relevant temperature of the plastic film to rise considerably above the constant reflect temperature (300 deg. F). This led to the change and magnitude of experiments where this phenomenon was investigated. The temperature rise does not occur when pressure is not increased (Figure 14). Temperature related changes in the mechanical strengths of plastic film were investigated up to 35 deg. C. The shape of force-deformation curves show an expected softening of the material at high temperatures. The contents of the film included some air, and the temperature of the gaseous phase is expected to rise when the volume of the chamber vapor volume is reduced due to changes in pressure. To exclude this possibility, a gas pump under airtight compression and a reduced chamber volume were assigned.

adiabatic compression is described by the following equation [Weaver and Street, 1981]:

$$P_2/[(P_2/V_2)^{1/\gamma}] = P_1/[(P_1/V_1)^{1/\gamma}] \quad (10)$$

where

- P_1 and P_2 are the absolute internal pressures before and after squeezing, respectively,
- V_1 and V_2 are the corresponding absolute volumes,
- R is the universal gas constant, and
- γ is the adiabatic exponent for air (1.4).

Calculations with this equation showed that a temperature rise of 5 deg. F (3 deg. C) could be expected for one psi increase in internal pressure. Such a pressure increase logically could be expected from the facts that all return controls were operated manually and the mechanical resistances of both the gun and the fill would reduce volume changes to some extent. A computer program (Set-Line gun in Appendix "B") was designed to calculate the corresponding changes in temperature due to a pressure rise for saturated air (air-water vapor mixture). The results in this case showed an increase of 5 deg. F per psi. (3.0544C deg. C per Pa) (Weaver and Street, 1981).

The significance of these findings is that upon compression in plastic fills under the pressure conditions previously described, the fill experience greater adiabatic rise in the

processed product. This possibility could be addressed with rigid chlorine biodegradations. These results also appear to offer a direct corollary to the work reported by Tinsley et al., (1988), and Cleland and Ierabekian (1989) who investigated the water-cooling effect of sudden reject depressurizations. Some steps to reject pressure could cause less than the expected boiling to be achieved in a process.

The experiments performed using plastic and chlorine dips were very significant because a) the relative heat transfer properties could not replace the persistent discrepancies between microbiological and calculated values of the number of bacteria spores and b) the chlorine dips closely followed the thermal history of the contents of a can and provided mechanical protection in case of a sudden pressure rise.

Experiments in Kinetics

Parameters involved in the response formulas (Table 1) were determined, and the efficacy of the response formulas in fitting experimental data was tested using a series of historical experiments. Subsequently, the model was validated by comparison of predicted and experimental responses for a nonthermal operation of the sterilization system.

Isolated Experiments for parameter determination

The number of surviving isolated spores as a function of time for treatments at four constant temperatures are presented in Figures 16, 17, 18 and 19. Recent control points at the beginning of the experiments were hidden by the vertical axis, the origin of the time axis has been slightly displaced in these figures. The temperatures of the treatments were 47, 55, 66, and 75 deg. C. For each temperature, two curves are presented: one represents the survivors found by incubation at a temperature of 46 deg. C, and the other corresponds to survivors found by incubation at 32 deg. C. The general trend is for the low curves to begin to look alike at high temperatures and for spores incubated at 32 deg. C, initially constituting a very small number, to become the dominant force over time. With the exception of the treatment at 47 deg. C, when enough time has elapsed, the number of injured survivor spores is larger than the number of survivors incubated at 46 deg. C. This trend can best be seen by viewing all four cases simultaneously, as in Figure 20.

For all experiments, activities surpassed the initial stages of heat treatment, due to large initial increments in the number of survivors. However, at higher temperatures, samples could not be taken sufficiently fast to demonstrate the rapid increments in the number of survivors because of the lagging

Figure 34. Survival curves for the treatment of tumors of R. rattus at 60 deg. C, including the curve for injected tumors that are resistant to irradiation at 10 deg. C and normal organs irradiated at 40 deg. C.

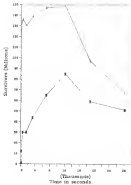


Figure 12. Survival curves for the treatment of spores of *B. subtilis* at 55 deg. C, including the curve for spores spores that are resistant to incubation at 55 deg. C and normal spores incubated at 55 deg. C.

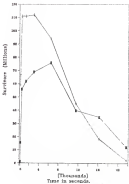


Figure 18. Survival curves for the treatment of sprays of *A. baumannii* at 10 day, C, including the curve for injured sprays that are killed by incubation at 30 day, C and normal sprays incubated at 40 day, C.

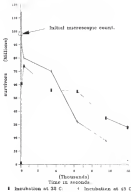


Figure 18. Survival curves for the treatment of spores of *B. subtilis* at 50 deg. C, including the curve for injured spores that are retained by incubation at 37 deg. C and normal spores incubated at 45 deg. C

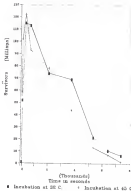


Figure 26. The four sets of survival curves together. The breakdown temperatures are 10 deg. C, b) 10 deg. C, c) 10 deg. C, d) 90 deg. C.



1. Temperature at 60 Hz

2. Temperature at 60 Hz



3. Temperature at 60 Hz

4. Temperature at 60 Hz



5. Temperature at 60 Hz

6. Temperature at 60 Hz



7. Temperature at 60 Hz

8. Temperature at 60 Hz

of the experimental procedure. It failed that accuracy of measurement during the first seconds of each experiment was limited. Consequently, the possibility of an initial transient occurring near the beginning of a test such as can be seen in Figure 18, could not be confirmed at the higher temperatures (Figure 15). In summary, the failed test is determined by activation, and it occurs shorter at higher treatment temperatures.

The exponential term in the response formula that accounts for the less heat-resistant population (β_0) of the spore suspension was not significant in this research because the spore suspension used was homogeneous for the following two reasons. One, the spore suspension used was more than three years old, free of vegetative cells and protozoa spores. Two, the organism tested did not present multiple phases (Lundberg, 1984).

The experimental data were analyzed using nonlinear regression to determine values of the parameters in equation 2. Calculated values for the carrier spores are presented together with corresponding experimental data in Figures 11, 21, 25, 26, 28, 30, 37 and 38. The graphs were prepared to represent comparison of calculated values and experimental data. Note that the scales are different in each case. The numbers presented were multiplied to minimize round-off error during the calculations. Time is divided by 15, not the number of carriers

Figure 11. Comparison between calculated turnover ratios and experimental data for species of *B. subtilis* exposed to heat treatment at 121 °C for 15 min incubated at 30 days - 2

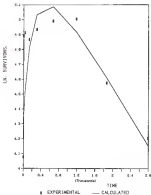


Figure 10: Comparison between calculated survival curves and experimental data for injured B. subtilis spores exposed to heat. Data from eq. 17 and eq. 18.

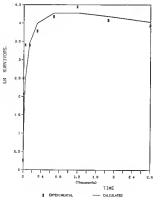


Figure 23: Comparison between calculated survival curves and experimental data for spores of *B. subtilis* exposed to heat treatment at 55-60°C and incubated at 45 deg. C

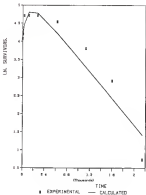


Figure 24 Comparison between calculated survivor curves and experimental data for injured L. polilla, squares represent the total treatment at 15 mg/L

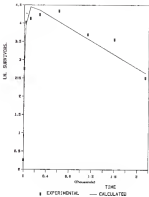


Figure 28: Steady-state behavior calculated surrogate cycles and experimental data for approx. 40 μ -methyl-1,2-cyclohexadiene to meet treatment at 40 deg. C and breakdown at 45 deg. C

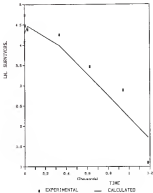


Figure 16. Comparison between calculated survival curves and experimental data for injured A. nubilus neurons exposed to heat. Generated at 16 May '94.

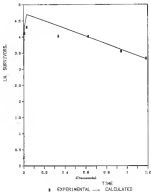


Figure 22: Comparison between calculated survival curves and experimental data for spores of *B. subtilis* exposed to heat treatment at 10 deg. C and incubated at 40 deg. C

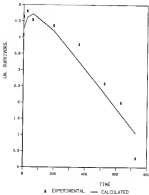
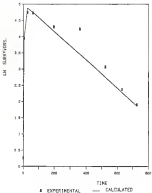


Figure 10: Comparison between calculated survival curves and experimental data for injured *E. coli* strains exposed to heat (Experiment 10, 10 deg. C).



by one unit). A natural logarithmic presentation was chosen in agreement with the exponential nature of the mathematical model.

Nonlinear Curve-Fitting

A computer program (NOLFIT.pas in Appendix "B") was developed for nonlinear least-square curve fitting of the data. Initial parameters were determined from graphs of the sparse suspension taken at the beginning of each experiment and arithmetic means were used for the calculations. 1.5×10^{-3} for the sparse suspended at 45 deg. C and 1.5×10^{-4} for the injured sparse; the standard deviations were 1.7×10^{-3} , and 1.7×10^{-4} correspondingly. The error (standard deviation expressed as a percentage of the mean value) was estimated from four replicas of one point on the survival curve to be 20% and 20% of the mean for the injured sparse. Estimation of the error is important in the nonlinear regression because the parameter minimized in the procedure is the square, and a good estimation of the error facilitates the curve fitting process. Increasing the standard deviation did not affect the values of the constants determined up to $\pm 100\%$ of the value of the mean. Also, the rate of convergence subjectively estimated by the computations time did not change

significantly. Low susceptibility of the nonlinear curve fitting process to error affected only a few experimental points.

The same procedure applied to data from Elmhurst population did to give all predicted by Dr. Lj. Matković tested the part of the model that accounts for a less heat-resistant population. The results are presented in Figure 25, and the good correspondence between the values calculated with the response function and the experimental data is obvious. In this case, the treatment temperature was 128 deg. C.

A scatter diagram of all corresponding calculated and experimental values is presented in Figure 26. The straight line represents the theoretical case of perfect correlation between predicted and actual responses. The regression equation (not presented in the graph) is

$$\ln y = 0.964412 \ln x + 0.8381 \quad (26)$$

where y and x represent calculated and experimental values, and the correlation coefficient (r) is 0.9644 with 26 degrees of freedom. The value very close to 1 and the very small intercept confirm the efficacy of the proposed model in describing the dynamics of spore populations under constant initial temperature treatment. A region of some discrepancy is found in the initial moments, as can be seen in Figures 21 and 25; this region should

Figure 28 Comparison between calculated values
and experimental data for
Chlorination inhibition of A. in olive oil
[Data taken from: J. P. Prost, 1991
G. Marnett, 2001]

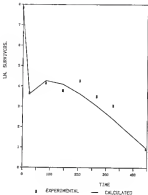
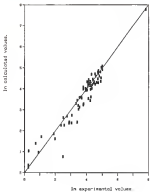


Figure 36. Scatter diagram showing correlation between experimental and calculated values for a total of 18 separate sets of experimental conditions.



be the subject of additional research: high temperature-short time processes may benefit from a better understanding of the events that occur during the first critical moments of exposure to lethal temperature.

Temperature dependence of rate constants. The rate constants of activation and deactivation for the standard case and for spores incubated at 33 deg. C are presented in figures 13, 14, 15, and 16. The graphs follow the form of the Arrhenius¹² equation for temperature dependence of rate constants, where the natural logarithm of the rate constant is plotted vs. the reciprocal of the absolute temperature ($1/T$). Activation energy (E_a) was calculated from the slope of the regression equation for a graph of the natural logarithm of a rate constant as a function of the reciprocal of absolute temperature. The regression equations are as follows:

For the spores incubated at 46 deg. C,

$$\ln k_a = - 27281 / RT + 61.4417, \quad r = + 0.9985 \quad (27)$$

$$\ln k_d = - 17844 / RT + 41.3268, \quad r = - 0.9979 \quad (28)$$

For the spores incubated at 33 deg. C,

$$\ln k_a = - 24629 / RT + 61.16411, \quad r = + 0.9974 \quad (29)$$

$$\ln k_d = - 42647 / RT + 112.1216, \quad r = + 0.9982 \quad (30)$$

The degrees of freedom are two in all cases.

Figure 11. Arrhenius plot for the isomerization rate constant for species of Eq. (10b)(11) measured at 40 deg. C.

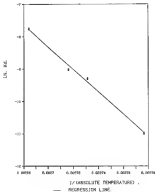


Figure 10: Aylward's plot for the inactivation rate constant of B. subtilis injured spores...

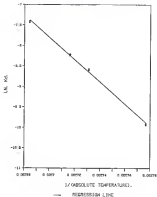


Figure 10. Arrhenius plot for the activation
rate constant for spores of *B. subtilis*
isolated at 48 deg. C

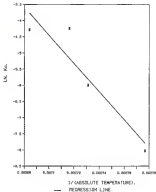
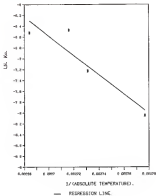


Figure 34. Arrhenius plot for the activation rate constant of H_2 reacting against species



The Arrhenius equation may be written as follows (Allen and Humphrey, 1971)

$$k = A^* \exp(-E_a/RT) \quad \text{or} \\ \ln k = \ln A^* - E_a/RT \quad \text{.....[90]}$$

The slope (p) of the curve is then

$$p = -E_a/R \quad \text{.....[91]}$$

The value of the universal gas constant, R , is 8.314 J/mole K (Gardner, 1977)

The activation energy is then

$$E_a = -pR \quad \text{.....[92]}$$

Values determined for the activation energy, in J/mole, are presented in Table 3.

Table 3- Activation energy (J/mole)

Inactivation ^a	1.05x10 ⁶
Activation ^a	1.44x10 ⁶
Inactivation ^{ab}	1.01x10 ⁶
Activation ^{ab}	1.42x10 ⁶

^a Incubation at 45 deg. C

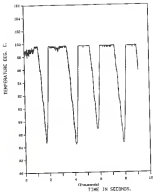
^{ab} Incubation at 30 deg. C

Dependence of the rate constant of leaching (k_p) for the two leaching temperatures of 45 and 55 deg. C is described very well by the Arrhenius equation, and the dispersion of the data is very low. Activation energy corresponds to E in the Eyring model. Determination of the collection constant is influenced by logistical limitations of the experimental procedure. The first two points in Figures 12 and 13, corresponding to higher leaching temperatures, show some disagreement. This discrepancy explained the need to explore the high temperature-short time region in order to provide better values for the constants in the Arrhenius equation describing the temperature-dependence of activation. Evaluation of leaching rate constants was not as accurate as predictions of the leaching rate constants, and the absolute value of the correlation coefficient was higher than 0.9.

Preliminary Rate-Controlling-Model-Independent Experiment

The model was tested by an experiment wherein the temperature of leach was held constant. The logical history of the leaching temperature used was recorded by computer and it is presented in Figure 14. The results support one of the leaching model over the traditional model. In order to compare

Figure 35. Time-temperature history for the diffusion coefficient experiment.



predictions of the model with the experimental data, average values of the initial population of each type of spore (obtained from parameter determination) were found to be as shown in Table 4.

Table 4. Average initial populations (in millions)

	Age	Standard deviation
Initially active spores, (30 deg. C) ^a	25.75	5.39
Dormant activation-requiring spores, (30 deg. C) ^a	125.75	41.32
Initially active spores, (35 deg. C) ^a	1.75	3.43
Dormant activation-requiring spores, (35 deg. C) ^a	22.25	24.75

^a Incubation temperature

The initial number of active spores identified by a low incubation temperature was, as expected, relatively small, while the initial number of dormant activation-requiring spores were higher for high incubation temperatures. The average initial populations will be more accurate when more trials are used and, thus, results from application of the model will improve. The initial populations obtained in the first experiments described

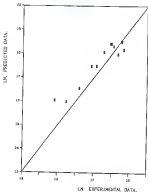
probably, probably due to the removal and use of the lower temperature populations in the tube that held the spare suspension or to adherence of the spores to the tube wall. The direct microscopic count was 5.2×10^6 and the standard deviation was 2.2×10^6 .

A computer program (written in C) was developed to calculate the number of surviving spores that result from a given temperature history (simulation) using the response model. The rate constants were calculated from the response equations for the sensitive formula, and the initial populations used were the averages shown in Table 4. The program calculated changes in the spore population for each time increment, and it used the new population obtained as initial values for the next time interval and recorded these in a computer file, together with the time in seconds.

A comparison of the number of surviving spores calculated from the response formulae and the values determined experimentally is presented in Figure 3b. The numbers are always of the same order of magnitude, but there are positive and negative deviations from the ideal case. This is a significant improvement over the results obtained using the traditional log-kill model.

The logarithmic nature of lethality values is very sensitive to changes in the value of k_{D10} . Values of 0 and ∞ can be used to show research was incapable to predict the kinetics of a

Figure 16. Comparison between predicted and experimental number of survivor spores for the microthermal experiment.



prediction of bacterial spores using Bigelow's model. Improved kinetic models describing inactivation of bacterial spores could play a major role in achieving more precise prediction of spore survival in a lethal heat treatment.

Discussion. Development of the mathematical model in this study was supported by a series of isothermal experiments, using *B. subtilis* spores, to determine values of the parameters involved and to verify the ability to respond properly to constant temperature treatment. The temperature dependence of rate constants also was determined, and a variable-temperature experiment tested the capability of the mathematical model with the temperature dependence of the rate constants incorporated.

Figure 10 presents four survivor curves together in order to facilitate comparison. Logical failures of the non-isothermal technique at the beginning of each experiment led to some uncertainty in the activation rate constants, particularly at higher temperatures (Figures 10 and 10'). The survivor curve for the 55 deg. C treatment may have resulted a somewhat lower value than it appears in the graph. This nucleus would be expected as a consequence of rate constants increasing with temperature; this idea is supported by the first two points of the 55 deg. C survivor curve.

Spored spores increase in relative importance with time. In general, at the end of each experiment they became the dominant

prediction of survival). The demand of injury in the long term emphasizes the importance of including it in a kinetic model. Injury may be particularly important in cases when storage conditions might favor the germination and outgrowth of injured spores.

The applicability of our model to describe changes in the number of survivor spores, as shown in Figure 10, for GL_10101 Type spores that included a heat-resistant population, emphasizes the potential of the approach followed in this work to handle complex situations that often occur in food engineering and the importance of dealing with these problems in more complexity. The first test, using a variable temperature process, can be considered successful because the characteristics found can be attributed to experimental error. This model should be used in order to achieve meaningful results with microbiological estimation of thermal sterilization processes. This improved model may also become a tool to work research, leading to an even more realistic mathematical characterization of bacterial spore behavior under treatment at better temperatures.

The need for an improved kinetic model is supported by the evidence of occasional reactions as reported in the literature. Reactions such as autolysis and injury are the possibility of alternative germination mechanisms and heat-resistant populations are not considered in Bigelow's model.

ter, all of these factors definitely influence the spore population surviving a thermal process. Special care has been taken to develop computer programs that permit application of the model by microcomputer, as a means of facilitating use of the model in the design and validation of thermal sterilization processes.

CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

Conclusions

The aluminum hydroxide-former with the highest, fabricated, and evaluated in this research was determined to be superior to plastic with used in commercial practice for validating thermal sterilization process. The superiority lies in the superior heat transfer and rigidity of the aluminum units. However, heat transfer and mechanics can not explain completely the large discrepancy often found between the number of spores surviving a given thermal history as determined by deger's kinetic model and by microbiological enumeration.

An improved mathematical model of a bacterial spore population under initial thermal treatment was developed and tested satisfactorily. The new model took into consideration those concomitant spore transformations (inactivation, activation and injury) as well as the presence of less heat-resistant fractions and the activation of alternative germination mechanisms.

The understanding of processes related to thermal inactivation of bacterial spores has been widened. The following statements summarize these insights:

1. Biphasic kinetic model is significantly superior for validation of commercial sterilization processes because it does not reflect the relevant biology.

2. Chitosan bioindicator units follow the thermal history of the materials of the can more closely than plastic units, and are less susceptible to pressure-induced temperature changes.

3. Spores analysis approach was successfully used to develop a model of a bacterial spore population with in the initial temperature range.

4. The transformations studied --activation, inactivation and injury-- followed pseudo-first order reaction kinetics within the time intervals used in the experiments, and they behaved as independent, reversible and irreversible reactions.

5. The mathematical model developed in this work closely described the dynamics of the number of survivor spores under treatment at constant initial temperature.

6. Arrhenius' equation conveniently describes the temperature dependency of the rate constants in the temperature range tested.

7. Using the Arrhenius equation to describe the temperature dependency of parameters in the mathematical model, the response formula predicted changes in the number of survivor and injured spores when the temperature was allowed to vary with time.

8. For the spore suspension tested, the number of spores capable of growing at 33 deg. C was higher than the number of

various agents determined by incubation at 45-deg. C in the final range of the time interval tested).

5. The experimental procedure adopted in this study requires small sample sizes, is simple to use, and yields good results for temperatures up to the boiling point of water. It also circumvents many of the shortcomings of traditional TSC and wet glass tubes to cell or tissue tubes.

20. The computer programs developed in this research for data analysis and simulation show the great potential of system analysis and micro-computers as tools in applied microbiology.

Suggestions for Future Work

The order of reaction would be studied carefully for the transformations considered in the model presented in this dissertation. Inspection of some data for the long term changes in Enteritis plausi (Kinsley, 1988) shows a departure from first order kinetics that can not be explained by a mixed population. A more accurate description of the kinetics may provide a better mathematical model. The following are specific suggestions:

1- The early events of heat treatment at initial temperature should be studied in more detail. Better experimental equipment, allowing faster sampling and higher treatment temperature, can be envisaged following basic concepts of the

apparatus used in this work... computer control and data acquisition, injection of a small sample of concentrated sperm suspension into an already equilibrated and well mixed reservoir, and heating of the sample at low temperatures. The new challenge is a very fast sampling procedure that prevents condensation across large pressure differences.

2. The procedure followed in this work should be applied to study recovery of sperm from injury and inactivation related to long-term storage of animal products.

3. In general, population dynamics and system analysis should be used for the solution of complex fluid engineering problems.

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APPENDIX B

COMPUTER PROGRAM TO CALCULATE SATURATION TEMPERATURE FROM
SATURATION PRESSURE FOR AIR-WATER MIXTURES

```

program satline;
(*calculates saturation temperatures from saturation pressure,
see formula 3.2.2, page 26 of the 1981 ASHRAE Book of
Standards.*)

(*valid for 0.05 to pressure to 840 psi*)

const
  atm=14.7;

var
  n = array[1..10] of real; (*coefficients for the
                             formula*)
  i = integer;
  name,
  pressure,
  temperature = real;
  fin = text; (*input file*)

function power(x:real;exponent:integer):real;
begin
  power:=exp(exponent*ln(x));
end; (*power*)

begin
  name:=0;
  assign(fin,'satline.dat'); (*file with the values
                             for the coefficients*)
  reset(fin);
  for i:=0 to 9
  do readln(fin,a[i]);
  writeln('load the saturation pressure in psi');
  readln(pressure);

  for i:=0 to 9 do
    name:=name+a[i]*power(n[i],ln(pressure/a[i]));
    temperature:=name/100;
  writeln;
  writeln('the saturation temperature is: ',temperature:0:1,'
    deg. F. ');

  close(fin);
end; (*sat line*)

```


[*The $\text{MPL}_{\text{DSE}}^{\text{DSE}}$ is set with the value of the $\text{coeff}(k)$ term*]

```

DO (K=1)
  P4=5000
  E=0.001
  +E=2473447
  E=751444
  +E=811000
  E=8839+100
  +E=2499440-1
  E=6195110-0

```

APPENDIX B

COMPUTER PROGRAM FOR THE NONLINEAR REGRESSION ANALYSIS OF
DATA.


```

>         h[1,ncol,1] := h[ncol,1];
>         h[ncol,1] := dim
>     END
> END;
>
> hdiag[1] := 1/ncol;
> hdiag[1] := 1/col;
> IF (n[ncol,1] = 0.0) THEN BEGIN
>   writing('page 2 in GMSOL - singular matrix');
>   readln
>   BEGIN
>     g[1,ncol,1] := 1.0/h[ncol,1];
>     a[ncol,1] := 1.0;
>     FOR i := 1 TO n DO BEGIN
>       a[ncol,i] := a[ncol,i]*g[1,ncol,1];
>     END;
>     FOR i := 1 TO n DO BEGIN
>       h[ncol,i] := a[ncol,i]*g[1,ncol,1];
>     END;
>     FOR ii := 1 TO n DO BEGIN
>       IF (i = col) THEN BEGIN
>         dim := a[1,ncol];
>         a[1,ncol] := 1.0;
>         FOR i := 1 TO n DO BEGIN
>           a[1,i] := a[1,i]*a[ncol,i]/dim;
>         END;
>         FOR i := 1 TO n DO BEGIN
>           h[1,i] := a[1,i]*a[ncol,i]/dim;
>         END;
>       END
>     END
>   END;
>   FOR i := n DOWNTO 1 DO BEGIN
>     IF (hdiag[i] < hdiag[1]) THEN BEGIN
>       FOR ii := 1 TO n DO BEGIN
>         dim := a[i,hdiag[1]];
>         a[i,hdiag[1]] := a[i,hdiag[i]];
>         a[i,hdiag[i]] := dim;
>       END;
>     END
>   END
> END;
> PROCEDURE gmsol[1] RETURNS g[1:ncol, 1:n] INTEGER, a[1:ncol, 1:n]
>   LOCAL g[1:ncol, 1:n] INTEGER;
>   /* Reverse-lookup routine GMSOL must define the types
>   FOR
>   g[1:ncol] = ARRAY [1..ncol,1..n] OF REAL;
>   g[1:ncol] = ARRAY [1..ncol] OF INTEGER;
>   In the calling program. */

```

```

> END
>
> 1.1) Integer,
> beta, real,
> BEGIN
>   FOR j := 1 TO m-1 DO BEGIN
>     FOR i := j+1 TO m DO BEGIN
>       covar[i,j] := 0.0
>     END
>   END
>   FOR i := 1 TO m-1 DO BEGIN
>     FOR j := i+1 TO m DO BEGIN
>       IF (listag[i] = listag[j]) THEN BEGIN
>         covar(listag[i],listag[i]) := covar[i,i]
>       END ELSE BEGIN
>         covar(listag[i],listag[j]) := covar[i,j]
>       END
>     END
>   END
>   covar(listag[1],listag[1]) := cov;
>   FOR j := 2 TO m DO BEGIN
>     covar(listag[j],listag[j]) := covar[i,j]
>   END
>   FOR j := 2 TO m DO BEGIN
>     FOR i := 1 TO j-1 DO BEGIN
>       covar[i,j] := covar[j,i]
>     END
>   END
> END
>
> PROCEDURE ansatz(x,y,ang: glodata; ndata: integer);
>   var a: glom; m, n: integer; lista: glilista;
>   alpha: integer; bdd: alpha: glaliqua;
>   var beta, glom, nalg: integer; bdd: chom, real;
> /* Program using routine MINIM that provides a
> MINIMIZE function, a glom, pff: real; dydg,
>   m: integer;
> that evaluates the fitting function pff and its derivatives
> dydg
> with respect to the parameters a at point m. Also they
> must define the types
> [1]
>   glodata = ARRAY (1..ndata) OF real;
>   glom = ARRAY (1..m) OF real;
>   glilista = ARRAY (1..m) OF integer;
>   glaliqua = ARRAY (1..nalg,1..nalg) OF real;

```

```

+ to the main routine *)
+
+
+  k,j:= integer;
+  y:=0;g:=ginit;dy:=real;
+  dy:= glens;
+
+  BEGIN
+    FOR j := 1 TO nfit DO BEGIN
+      FOR k := 1 TO j DO BEGIN
+        alpha[j,k] := 0.0
+      END;
+      beta[j] := 0.0
+    END;
+    chiq := 0.0;
+    FOR i := 1 TO nvars DO BEGIN
+      funcg[i]:=a*(y+ydy*(j-1));
+      ydy:=1.0/(y+ydy*(j-1));
+      dy:= ydy*(ydy);
+      FOR j := 1 TO nfit DO BEGIN
+        y:= dy*(funcg[i]**ydy);
+        FOR k := 1 TO j DO BEGIN
+          alpha[j,k] := alpha[j,k]+ydy*(funcg[i]**k)
+        END;
+        beta[j] := beta[j]+ydy*y
+      END;
+      chiq := chiq+ydy**ydy*ydy
+    END;
+    FOR j := 1 TO nfit DO BEGIN
+      FOR k := 1 TO j-1 DO BEGIN
+        alpha[k,j] := alpha[j,k]
+      END;
+    END;
+  END;
+
+  FUNCTION arqin(x,y,z) GLISTS, nvars: integer;
+  VAR ai: GLISTS; nfit: integer; beta: GLISTS;
+  wfit: integer; var qvar, alpha: GLISTERS;
+  nfit: integer; VAR chiq, almdat: real;
+
+  (* Program using routine ARQIN must define the type
+  *)
+  GLISTS = ARRAY [1..nvars] OF real;
+  GLIST = ARRAY [1..nfit] OF real;
+  GLISTS = ARRAY [1..nfit] OF integer;
+  GLISTERS = ARRAY [1..nfit,1..nvars] OF real;
+
+  and the variables
+  VAR
+    glenfit: real;
+    glenr, glenat: glens;
+
+  to the main routine. Also note that this routine calls ARQIN,
+
+  which

```



```

* requires a user-defined procedure FUNC, described in the
* routine.  *)
* [LAMB, 05.]
* END
*
* k, n0, j, n11, i0n0p, i0n0q,
* do: gfunc;
* write: gfuncname;
* BEGIN
*   IF (n0n0 = 0.0) THEN BEGIN
*     n0 := n0n0+1;
*     FOR j := 1 TO n0 DO BEGIN
*       n11 := 0;
*       FOR i := 1 TO n11 DO BEGIN
*         IF (i0n0p) = j) THEN n11 := n11+1
*       END;
*       IF (n11 = 0) THEN BEGIN
*         i0n0q[i0] := j;
*         n0 := n0+1
*       END ELSE IF (n11 = 1) THEN BEGIN
*         write:('Case 1 in routine MGRM');
*         write:('Improper permutation in L12M'); read:
*       END;
*     END;
*     IF (n0 = (n0n0-1)) THEN BEGIN
*       write:('Case 2 in routine MGRM');
*       write:('Improper permutation in L12M'); read:
*     END;
*     n0n0 := 0.001;
*   END
*   MGRM(a, n, n0, n0n0, j, n0, i0n0, n11, alpha, gfunc, n0, n11);
*   gfunc := gfunc;
*   FOR j := 1 TO n0 DO BEGIN
*     gfunc[j] := a[j]
*   END;
*   END;
*
*   FOR j := 1 TO n11 DO BEGIN
*     FOR i := 1 TO n11 DO BEGIN
*       cfunc[i, j] := alpha[j, i]
*     END;
*     cfunc[j, i] := alpha[j, i]^2/(1+a*n0n0);
*     sfunc[j, i] := gfunc[i, j]
*   END;
*   (cfunc) := cfunc, n11, n0, sfunc, j, i;
*   FOR j := 1 TO n11 DO sfunc[j] := sfunc[j, j];
*   IF (n0n0 = 0.0) THEN BEGIN
*     cfunc := cfunc, n0, n0, i0n0, n11;
*     write: ();
*   END;

```



```

% For i=1 to numel
%
%   @ write(fout,'a[',i,'] = ',a(i)/R);
%
write(fout);
write(fout,'The conversion ratio is: ');
for j=1 to numel @
    @
    for j=1 to numel
        @ write(fout,conv(1,j)/R);
        write(fout);
    end;
end;

write(fout);
write(fout,'The curvature matrix is: ');
for i=1 to numel @
    @
    for j=1 to numel
        @ write(fout,ctype(1,j)/R);
        write(fout);
    end;
end;

write(fout);
write(fout,'That is all there is, there are no more
');
% close(fout);
% end;

%=====
%Open File%
Filename='test02.dat';
write(fout,Filename);
read(fout);
read(fout,numel,ctype);
%initialise arrays%
for i=1 to numel @
    @
    x(i)=0;
    y(i)=0;
    z(i)=0;
    for j=1 to numel @
        @
        conv(1,j)=0;
        ctype(1,j)=0;
    end;
end;
end;
%initialise variables%
ctable=0; %Integer value for the
          %initialisation of the

```


APPENDIX C

COMPUTER PROGRAM TO CALCULATE THE NUMBER OF SURVIVOR SPREADS FROM A FILE CONTAINING THE TIME-CONCENTRATION HISTORY, USING THE MATHEMATICAL MODEL DEVELOPED


```

is the next time interval?')

constants nfa(averageTemperature)-Nfa(averageTemperature),
          constant(nfa(averageTemperature));

(*Prepends for the calculation of the conversion
for the next time interval*)

if (time()-startIntervalTime(1-1) <= 1*60) then
  nextIntervalTime(1)-0/0 0 1/60
  nextIntervalTime(1)-constant(1)
  nfa-constant(1)useInteger(1);
(*to prevent underflow when time is long*)

actIntervalTime(1)-constant(1)exp(-constant(1)useInterval)-
  constantIntervalTime(1);

writeLn('Time,Time(1):',15:0,' ');
actIntervalTime(1)-1/60(1);
(*write output file*)
writeln('Time');

end;(*actIntervalTime*)

procedure classification;
begin
  Ctime(1);
  Ctime(2);
  Ctime(3);
end;(*classification*)

begin
  specification;
  startIntervalTime;
  calculateIntervalTime; (*also writes output file*)
  Ctime(1);
  end;(*main loop*)

```


BIOGRAPHICAL SKETCH

Alfredo C. Rodríguez Domínguez was born in Mexico City, Mexico, where he attended primary school, junior high and high school. He received the degree of Mechanical Engineer in 1971, and the degree of Master of Science in 1978 from the National School of Integrated Sciences of the National Polytechnic Institute.

His professional experience is in the field of the food sciences. He has been in charge of several research, development and innovation projects. Before starting graduate studies in the University of Florida, he was head of the Department of Program Evaluation and Internationalization Cooperation within the Graduate School of the National Polytechnic Institute in Mexico.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


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